REMARKS

Applicants have carefully studied the Office Action mailed on August 9, 2002, which issued in connection with the above-identified application. The present amendments and remarks are intended to be fully responsive to all points of rejection raised by the Examiner and are believed to place the claims in condition for allowance. Favorable reconsideration and allowance of the present claims are respectfully requested.

Restriction/Election

Claims 1-41 were pending and at issue in the application. In the Office Action, the Examiner made final the election of Group II (claims 15-32 and 39) and withdrew claims 1-14, 33-38, and 40-41 from consideration. In the Office Action, the Examiner indicated that the claims of Group II and Group I (claims 1-14) could be rejoined if the claims of Group I were limited to the plasmids with inserts recited in the claims of Group II. Claim 1 has been canceled and the remaining claims have been amended as specified by the Examiner to recite the plasmid inserts recited in the claims of Group II. It is therefore respectfully submitted that claims 2-14 should be rejoined with claims 15-32 and 39 and examined in the present application.

Pending Claims

Claims 1-41 were pending and at issue in the application. Claims 15-32 and 39 have been rejected under 35 U.S.C. § 112, first and second paragraphs, for lack of enablement and as being indefinite. Claims 15-32 and 39 have been also rejected under 35 U.S.C. § 103(a) as being obvious over the prior art.

Claims 33-38, 40 and 41 have been canceled without prejudice as drawn to a non-elected invention. Claim 1 has been canceled without prejudice or disclaimer. Claims 5 and 15 have been amended and their new respective dependent claims 42-43 and 44-45 have been added in order to more particularly point out and distinctly claim the invention. Specific support for the

newly introduced recitations "a regulatory element for the synthesis of vRNA or cRNA with the exact 3' end" and "wherein the regulatory element for the synthesis of vRNA or cRNA with the exact 3' end is a ribozyme sequence" can be found, for example, at p. 14, ll. 22-29 and p. 27, ll. 18-25 of the present specification[1]. Support for the recitation "thereby resulting in expression of vRNA or cRNA" in claim 15 can be found, for example, at p. 10, ll. 3-10; p. 11, ll. 14-24; p. 13, ll. 7-15; p. 16, ll. 13-18; p. 20, ll. 1-21; p. 27, l. 25 - p. 28, l. 8; Example 5, and Figures 1, 5 and 9. Claim 5 has been also rewritten in independent format and amended following the Examiner's suggestion to allow the rejoinder and examination of claims 2-14. Claims 2-4 and 16-17 have been amended to correct dependency. Claims 8, 12, 18, and 21-22 have been amended to correct formal defects. No new subject matter has been added as a result of these amendments; no new search is required, and no new issues are raised. Upon entry of these amendments, claims 2-32, 39 and 42-45 will be pending.

35 U.S.C. §112, Second Paragraph, Rejections

In the Action, claims 15-32 and 39 have been rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. Specifically, the Examiner contends that the term "autonomous" in claim 15 is unclear. As claim 15 has been amended to delete this term, the rejection is rendered moot.

The Examiner also states that the term "minimum" in claim 15 is a relative term that lacks a comparative basis and is therefore unclear. Applicants respectfully disagree and note that a detailed and specific definition of this term is provided at p. 29, ll. 16-24 of the present specification. According to this definition, a minimum plasmid-based system is the system

[1] Page and line numbers provided in the present response correspond to the substitute specification filed on January 9, 2002.

having the total number of plasmids that does not exceed the total number of gene segments from the source RNA virus. In fact, in the last three sentences at p. 4 (continuing at p. 5) of the Office Action the Examiner himself states that he understands the term "minimum" to mean precisely as defined at p. 29, ll. 16-24 of the specification.

The Examiner further objects to the use of the word "are" in line 6 of claim 15. In response, applicants respectfully note that the term "are" refers to several sequences (*i.e.*, viral cDNA corresponding to viral genomic segment, pol I promoter, and a regulatory element for the synthesis of vRNA or cRNA with the exact 3' end), which are inserted between pol II promoter and a polyadenylation signal.

The Examiner's objection at p. 3 of the Office Action to the terms in lines 2-3 of claim 15 is believed to be without merit. Regarding the composition of the cloned viral cDNA recited in claim 15, applicants respectfully note that each plasmid constituting the minimum plasmid-based system of the instant invention comprises a viral cDNA corresponding to one viral genomic segment.

In the Action, the Examiner also objects to the use of the phrase "having a map selected from" in claims 23 and 24. It is respectfully submitted that the phrase "having a map" is not indefinite and means that the encompassed plasmids are organized like the recited specific plasmids pHW241-PB2, pHW242-PB1, pHW243-PA, pHW244-HA, pHW245-NP, pHW246-NA, pHW247-M, pHW248-NS, pHW181-PB2, pHW182-PB1, pHW183-PA, pHW184-HA, pHW185-NP, pHW186-NA, pHW187-M, or pHW188-NS, but are not necessarily the same. In other words, these plasmids contain the same sequences and distribution of pol I promoter, a regulatory element for the synthesis of vRNA or cRNA with the exact 3' end (*e.g.*, pol I terminator or ribozyme sequence), pol II promoter, polyadenylation signal, selection marker, replication system, etc. (*see* the definitions and descriptions provided at p. 42, ll. 11-19, p. 55, l. 16 - p. 57, l. 13 and Figures 3A-B of the present specification). There is no doubt that a skilled artisan would understand the claim to embrace plasmids with the same relative spatial

arrangement of specific functional elements of the regulatory and structural sequences, since such maps are commonly provided in publications with the understanding that the skilled artisan could reproduce the functionally identical plasmid, whether or not certain structural features are identical.

In light of the foregoing, applicants respectfully submit that the rejection of the claims based upon 35 U.S.C. §112, second paragraph, is overcome and withdrawal of such is kindly requested.

35 U.S.C. §112, First Paragraph, Rejections

In the Action, claims 15-32 and 39 have been rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for plasmid-based systems for production of influenza viruses, allegedly does not reasonably provide enablement for plasmid-based systems for production of the full range of negative stranded viruses, in particular, non-segmented negative strand RNA viruses and negative strand RNA viruses replicating in the cytoplasm.

Applicants respectfully traverse the rejection and note that, in contrast to the Examiner's assertion, methods and plasmid-based systems for production of negative strand RNA viruses other than influenza A viruses (including viruses with segmented and non-segmented genome, viruses replicating in the nucleus and viruses replicating in the cytoplasm) are disclosed, for example, at p. 14, l. 6 - p. 15, l. 14; p. 19, l. 18 - p. 22, l. 10; p. 23, ll. 3-13; p. 23, l. 24 - p. 25, l. 19; p. 28, ll. 13-22; p. 29, ll. 1-8; p. 31, ll. 8-22; p. 76, l. 3 - p. 77, l. 4, and Figures 7A-B, 9, 10, and 11A-C. Methods and plasmid-based systems for production of specific negative strand RNA viruses other than influenza A viruses are disclosed in detail, for example, in the following parts of the instant application:

a) influenza B virus (*Orthomyxoviridae*, eight-segmented negative strand RNA virus): p. 64, ll. 20-23 and Example 3 (pp. 65-66);

b) influenza C virus (*Orthomyxoviridae*, seven-segmented negative strand RNA virus), Thogotovirus (*Orthomyxoviridae*, six-segmented negative strand RNA virus): p. 64, ll. 20-24; p. 76, ll. 3-6;

c) *Arenaviridae, Bunyaviridae* - p. 64, ll. 24-27; p. 76, ll. 6-11;

d) parainfluenza virus III (*Paramyxoviridae*, non-segmented negative strand RNA virus): p. 16, l. 13 - p. 17, l. 8; p. 22, l. 11 - p. 23, l. 2, and Figure 9;

e) viruses with segmented double-stranded RNA genomes (*Reoviridae* [Rotavirus, Orbivirus, Orthoreoviruses], *Birnaviridae*): p. 17, l. 9 - p. 19, l. 2 and Figure 10.

Also, similarly to the present specification, in the article in the *Journal of General Virology* (Hoffman and Webster, J. Gen. Virol., 2000, 81: 2843-2847), which is incorporated in the present application by reference in its entirety (*see* p. 71, ll. 16-17), the inventor and co-workers state (p. 2846, left col., last ¶):

> The high efficiency of the eight-plasmid system for the generation of influenza A virus indicates that this system should be applicable to other orthomyxoviruses, e.g. influenza B virus, influenza C virus and Thogoto virus. The results in this study suggest that the vRNA-mRNA system will be the most efficient way for generating these viruses entirely from plasmids. A different challenge is the establishment of reverse genetics-based systems for the generation of RNA viruses other than members of the family *Orthomyxoviridae*, e.g. members of the *Paramyxoviridae*, *Arenaviridae* or *Bunyaviridae*. Unlike orthomyxoviruses, most RNA viruses replicate in the cytoplasm of infected cells... Use of the pol I transcription system for cytoplasmic RNA viruses would require that the RNA transcripts have to be transported out of the nucleus. That pol I transcripts are indeed transported out of the nucleus is supported by the detection of protein production in cells containing pol I transcripts that had an internal ribosomal entry site inserted into the 5' noncoding region.

Applicants respectfully submit that the Examiner has failed to provide any reasons for non-enablement of the present disclosure with respect to plasmid-based systems for production of the full range of negative stranded viruses. Accordingly, the Examiner has failed to establish the prima facie case of non-enablement and to shift the burden to the applicants to demonstrate the enablement. As stated in MPEP Section 2164.04 (emphasis added):

> In order to make a rejection, the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) (examiner must provide a reasonable explanation as to why the scope of protection provided by a claim is not adequately enabled by the disclosure). A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support... *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). As stated by the court, "it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." 439 F.2d at 224, 169 USPQ at 370.
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> According to *In re Bowen*, 492 F.2d 859, 862-63, 181 USPQ 48, 51 (CCPA 1974), the minimal requirement is for the examiner to give reasons for the uncertainty of the enablement. This standard is applicable even when there is no evidence in the record of operability without undue experimentation beyond the disclosed embodiments. See also *In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995)...
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> ... the examiner should specifically identify what information is missing and why one skilled in the art could not supply the information without undue experimentation. See MPEP §2164.06(a)(a). References should be supplied if possible to support a prima facie case of lack of enablement, but are not always

required. *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). However, specific technical reasons are always required.

A number of recently published reports provide a further proof of enablement of the present disclosure with respect to the full range of negative stranded viruses by demonstrating the direct application of this system (as disclosed in the instant specification) for the generation of negative strand RNA viruses other than influenza A viruses. Specifically, the inventor and co-workers have recently published an article demonstrating that the eight-plasmid pol I-pol II system can be used for the generation of high yields of influenza B virus (Hoffmann *et al.*, Proc. Natl. Acad. Sci. USA, 2002, 99: 11411-6, attached as Exhibit A; see also Jackson *et al.*, J. Virol., 2002, 76: 11744-11747, attached as Exhibit B). As disclosed in detail at p. 11412 of the Hoffmann *et al.* article (left col., ¶2), influenza A and B viruses produce several different gene products and have significant differences in their genetic organization and sequences of homologous genes. The same article (p. 11416, left col., ¶1) also provides a suggestion to use a minimum seven-plasmid system for the generation of influenza C virus and a minimum six-plasmid system for the generation of Thogoto virus (THOV), which is a tick-transmitted orthomyxovirus with a genome consisting of six negative stranded RNA segments. This suggestion is based on a recent report by Wagner *et al.* (J. Virol., 2001, 75: 9282-9286, attached as Exhibit C), in which an efficient recovery of a recombinant THOV was achieved following transfection of 293T cells with 12 plasmids: six expression plasmids producing viral structural proteins by means of a vaccinia virus expressing the T7 RNA polymerase and six plasmids under the control of the pol I promoter producing vRNAs. Finally, in a recently published article by Flick and Pettersson (J. Virol., 2001, 75: 1643-1655, attached as Exhibit D; *see* also Flick *et al.*, J. Virol., 2002, 76: 10849-10860, attached as Exhibit E), the use of the plasmid-only-based system has been described for the generation of the members of the *Bunyaviridae* family, which have tripartite negative strand genome and replicate in the cytoplasm. As a model virus, the

authors used Uukuniemi (UUK) virus, a member of the *Phlebovirus* genus. Reporter cDNAs encoding CAT or GFP flanked by the terminal sequences of the UUK M RNA segment were transcribed in the cell nucleus by pol I, transported to the cytoplasm, and transcribed and amplified by the RNA polymerase L in the presence of the nucleoprotein N provided either from expression plasmids or by superinfection with UUK virus. At p. 1651 (left col.), the authors note:

> The fact that pol I reporter transcripts are noncapped and nonpolyadenylated raised the concern that these RNAs would not be efficiently transported out of the nucleus. In the case of influenza virus, the pol I transcripts do not have to exit the nucleus, since transcription and replication of vRNAs take place in the nucleus. In contrast, *Bunyaviridae* members replicate solely in the cytoplasm and the pol I transcripts therefore have to be exported from the nucleus. Our results showed that these concerns were unfounded, since CAT and GFP activities were readily detected. (emphasis added)

They further state at p. 1654 (right col., last ¶):

> The pol I system has recently been successfully developed to reconstitute infectious influenza virus entirely from cloned cDNAs (15, 20 [Hoffmann *et al.*, Virology, 2000, 267: 310-317], 31, 32). Our present results suggest that this could also be possible for *Bunyaviridae* members.

In view of the evidence provided above and the absence of any evidence that the claimed plasmid-based system will not work for any negative strand virus, it is believed that the Examiner imposes an overly high and burdensome duty on applicants, one not required by Section 112 or by the case law[2]. Thus, according to the current law and patent practice, the specification can permit some inferences to be drawn by those skilled in the art, and still comply with the enablement and written description requirement. In other words, there is no requirement that the claims be restricted to the working examples. Section 2164.03 of MPEP recites:

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[2] *See*, in particular, *In re Wands*, 858 F.2d 731-40, 8 USPQ2d at 1400-07 (Fed. Cir. 1988)

> the scope of the required enablement varies inversely with the degree of predictability involved, but even in unpredictable arts, a disclosure of every operable species is not required (*In re Vaeck*, 947 F.2d 488, 496 & n.23, 20 USPQ2d 1438, 1445 & n.23 (Fed. Cir., 1991); *In re Vickers*, 141 F.2d 522, 526-27, 61 USPQ 122, 127 (CCPA 1944); *In re Cook*, 439 F.2d 730, 734, 169 USPQ 298, 301 (CCPA 1971))

As further stated in section 2164.08 of MPEP:

> claims are not rejected as broader than the enabling disclosure under 35 U.S.C. 112 for non-inclusion of limitations dealing with factors which must be presumed to be within the level of ordinary skill in the art; the claims need not recite such factors where one of ordinary skill in the art to whom the specification and claims are directed would consider them obvious (*In re Skrivan*, 427 F.2d 801, 806, 166 USPQ 85, 88 (CCPA 1970))... When analyzing the enabled scope of a claim, the teachings of the specification must not be ignored because claims are to be given their broadest reasonable interpretation that is consistent with the specification.

See also *Application of Angstadt* (537 F.2d 498, 502-503, 190 USPQ 214, 218 [Cust. & Pat.App., 1976]) stating that applicants "are not required to disclose every species encompassed by their claims even in an unpredictable art." Similarly, in *In re Rasmussen*, court stated that "a claim may be broader than the specific embodiment disclosed in a specification" (650 F.2d 1212, 1215, 211 USPQ 323, 326 [Cust. & Pat.App., 1981]). Finally, in *In re Goffe* (542 F.2d 564, 567, 191 USPQ 429, 431 [CCPA 1976]), the court stated:

> To provide effective incentives, claims must adequately protect inventors. To demand that the first to disclose shall limit his claims to what he has found will work or to materials which meet the guidelines specified for "preferred" materials in a process such as the one herein involved would not serve the constitutional purpose of promoting progress in the useful arts.

In light of these standards and the above-presented arguments, it is believed that the present application provides an adequate enablement for plasmid-based systems for production of the full range of negative stranded viruses, including non-segmented negative strand RNA viruses and negative strand RNA viruses replicating in the cytoplasm.

Notwithstanding the foregoing, to expedite the prosecution, claims 5 and 15 have been amended to recite segmented negative strand RNA viruses. Applicants reserve the right to prosecute the broader subject matter in related continuing applications.

In the Action, claims 23 and 24 stand further rejected for lack of enablement of the recited specific plasmids. This rejection is respectfully traversed. Applicants note that claims 23 and 24 recite the plasmids having a map of pHW241-PB2, pHW242-PB1, pHW243-PA, pHW244-HA, pHW245-NP, pHW246-NA, pHW247-M, pHW248-NS, pHW181-PB2, pHW182-PB1, pHW183-PA, pHW184-HA, pHW185-NP, pHW186-NA, pHW187-M, or pHW188-NS. As specified above, the term "having a map of" means that the recited plasmids are organized like pHW241-PB2, pHW242-PB1, pHW243-PA, pHW244-HA, pHW245-NP, pHW246-NA, pHW247-M, pHW248-NS, pHW181-PB2, pHW182-PB1, pHW183-PA, pHW184-HA, pHW185-NP, pHW186-NA, pHW187-M, or pHW188-NS, but are not necessarily the same. Applicants further note that a detailed disclosure of how to make the claimed plasmids (including the sequences of the PCR primers) is provided in Examples 1 and 2 (in particular, at p. 55, l. 16 - p. 57, l. 13) and Figures 3A-B of the present specification as well as in the articles by Neumann *et al.* (Proc. Natl. Acad. Sci. USA, 1999, 96:9345) and Hoffmann *et al.* (Virology, 2000, 267:310 and Proc. Natl. Acad. Sci. USA, 2000, 97:6108) incorporated by reference in their entirety. Accordingly, it is believed that *no plasmid deposit is necessary* to support the claims and to allow one skilled in the art to practice the invention.

In light of the foregoing arguments, applicants respectfully submit that the rejection of claims based upon 35 U.S.C. §112, first paragraph, is overcome and withdrawal of such is kindly requested.

35 U.S.C. §103(a) Rejections

In the Action, claims 15-32 and 39 stand rejected under 35 U.S.C. §103(a) as being obvious over Hoffmann dissertation (1997) and Neumann *et al.* (Proc. Natl. Acad. Sci. USA, 1999, 96: 9345-9350). The Examiner contends that (i) at pp. 112-126, Hoffmann teaches a pol I-pol II plasmid expression system that can be used to express vRNA and mRNA and (ii) Neumann *et al.* teach the rescue of infectious influenza virus in the absence of helper viruses using co-transfection of 12 plasmids, 8 plasmids expressing vRNA segments and 4 plasmids expressing mRNA encoding support proteins. The Examiner concludes that one skilled in the art would have been motivated to use the plasmids of Hoffmann to make the rescue system of Neumann *et al.* more efficient, because it would require making fewer plasmids.

Applicants respectfully traverse the rejection and submit that, even if taken together, the cited references do not disclose or suggest the compositions and methods recited in the present claims and necessarily fail to provide a reasonable expectation of success in achieving the invention.

Legal Standards for Determination of Obviousness

The courts have held that where the prior art seeks to solve the same problem as the claimed invention but lacks significant elements of the claimed invention, it is improper to view the invention in a piecewise fashion to find its elements in the prior art. On the contrary, the invention must be viewed as a whole. *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540 (Fed. Cir., 1983); and *Phillips Petroleum Co. v. U.S. Steel Corp.*, 673 F. Supp. 1278 (D. Del., 1987). Even where the elements of the claimed invention are known, the claimed invention may still be patentable. *Gillette Co. v. S.C. Johnson & Son, Inc.*, 919 F.2d 720, 725, 16 USPQ2d 1923 (Fed. Cir., 1990). Further, it is improper to use hindsight to combine elements found in the prior art to reconstruct the claimed invention. *Gore*, 721 F.2d at 1552. In considering obviousness, the critical inquiry is whether something in the prior art as a whole

suggests the desirability, and thus the obviousness, of making a combination. *In re Newell*, 891 F.2d 899, 901-02, 13 USPQ2d 1248, 1250 (Fed. Cir., 1992). The Examiner must show some objective teaching from the art that would lead an individual to combine the references, *i.e.*, there must be motivation. In particular, the mere fact that the teaching of a reference may be modified in some way so as to achieve the claimed invention does not render the claimed invention obvious unless the prior art suggested the desirability of the modification (emphasis added). *In re Fritch*, 972 F.2d 1260, 23 USPQ2d 1780, 1783 (Fed. Cir., 1992) and see also *Ex parte Obukowicz*, 27 USPQ2d 1063 (Bd. Pat. App. & Intf., 1993). In other words, determination that the invention is obvious requires that (i) cited references teach the claimed invention as a whole, and (ii) both the suggestion of making the invention, and a reasonable expectation of success can be found in the prior art, not in the applicants' disclosure. MPEP Section 2143; *In re Dow Chemical Co.*, 5 USPQ2d 1529, 1531 (Fed. Cir., 1988); *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir., 1991).

The References Neither Teach Nor Suggest the Invention

Applicants respectfully submit that the Examiner's rejection does not meet the above-summarized legal criteria. Neither of the two cited references contains any suggestion or motivation to produce the minimum plasmid-based system of the present invention. The Hoffmann dissertation discloses that the use of pol I-pol II plasmids has to be combined with infection of a helper virus (FPV, *see, e.g.*, pp. 114-115), which provides viral polymerase proteins for transcription/replication of viral-like reporter RNA encoded by pol I-pol II plasmids. The Neumann *et al.* article does not teach either the pol I-pol II system or the use of a helper virus. It discloses a plasmid-only-based system comprising plasmids directing synthesis of vRNAs from a pol I promoter, which have to be co-transfected with pol II-driven protein expression (mRNA) plasmids encoding viral polymerase proteins. Taken for what the references fairly teach, the combination would provide a pol I-pol II system of Hoffmann in combination

with the plasmid-based system described by Neumann *et al.* to substitute for the helper virus. In other words, given the objective teachings of the references, without the benefit of hindsight gained from the instant disclosure, one might be led to substitute the Neumann plasmids for the Hoffmann helper virus. The references contain no further suggestion for modification.

The role of Neumann's pol II plasmids to replace helper virus is evident. Indeed, in explaining the importance of the advance made by Neumann *et al.*, Pekosz *et al.* note in their Comment to Neumann *et al.* article provided in the same issue of the *Proceedings* (Proc. Natl. Acad. Sci. USA, 1999, 96: 8804-8806, attached as Exhibit F, *see* p. 8804):

> In this issue of the *Proceedings*, Neumann and coworkers have come full circle on recovering recombinant, segmented, negative-strand RNA viruses with the production of influenza virus entirely from plasmid DNA and driven only by the host cell transcription and translation machinery... The lack of a helper influenza virus allows the virus from the initial transfection to be characterized immediately, thus limiting the chance of viruses containing reversions or second-site mutations from becoming significant contaminants.

Thus, if these references were artificially combined, they may only provide motivation to develop (i) a pol I-driven plasmid system utilizing a helper virus or (ii) a pol I-pol II system utilizing additional pol II-driven protein expression (mRNA) plasmids encoding viral polymerase proteins.

This analysis presupposes that there is some independent basis to combine the references. However, the Neumann plasmid-only-based system provides an alternative, not a modification, of the Hoffmann helper virus-based system. Nothing in Neumann suggests the desirability of substituting the pol II plasmids for Hoffmann's helper virus, nor does anything in Hoffmann provide a basis for using the pol I-pol II plasmids that express a non-viral reporter gene in the Neumann plasmid-based system. The very act of combining the disclosures of these two references requires hindsight gained from the application, since there is no objective basis in the references themselves for their combination.

There is No Reasonable Expectation of Successfully Achieving the Invention

The instant claims are directed to a pol I-pol II plasmid-only-based system for the generation of infectious negative strand RNA viruses from cloned viral cDNA, which involves construction and transfection of a total number of plasmids (each containing one copy of the viral cDNA) that does not exceed the total number of gene segments from the source RNA virus.

In contrast to the present invention, the Hoffmann dissertation does not disclose the application of the pol I-pol II system for the generation of an infectious virus, but merely describes its use in plasmids carrying heterologous reporter genes such as CAT and GFP. Furthermore, as specified above, in contrast to the present invention, the Hoffmann dissertation does not disclose a plasmid-only-based system but describes combining the transfection of pol I-pol II plasmids with infection of a helper virus. As explained at pp. 4-5 of the present specification (*see, e.g.*, p. 4, ll. 12-14 and ll. 27-29), the utility of the methods based on the use of a helper virus is limited, because a selection system is needed to obtain the desired virus from a vast background of the helper virus. Based on the disclosure of reporter gene expression provided in the Hoffmann dissertation, in the absence of hindsight based on experimental data and detailed disclosure provided in the present application, a person of ordinary skill in the art could not have concluded that the pol I-pol II system achieves sufficient expression and interaction of all of the viral components to produce an infectious virus, especially in the absence of any helper virus. In other words, transcription/replication of a single viral-like reporter RNA in the presence of viral polymerase proteins supplied by a helper virus (as disclosed in the Hoffmann dissertation) does not provide any teaching or suggestion of how all wild-type viral RNAs can be transcribed/replicated using pol I-pol II plasmids to achieve successful generation of infectious viral particles.

Moreover, the pol I-pol II system disclosed in the Hoffmann dissertation includes mutant regulatory sequences which have been introduced to increase the levels of transcription/ replication of reporter RNA. These mutant sequences include a "promoter up" mutation, which

differs from the wild-type sequence in 3 nucleotide positions at the 3' end of vRNA (G3->A3, U5->C5, C8->U8; *see* Figures 3-4 at p. 56 of the dissertation, *see* also Neumann *et al.*, Virology, 1994, 202:477-479 and Flick *et al.*, RNA, 1996, 2:1046-1057). It is well established (*see, e.g.*, Crescenzo-Chaigne *et al.*, Virology, 2002, 303: 240-252, attached as Exhibit G, ¶ bridging left and right col. at p. 248) that the increased levels of transcription/replication of viral-like RNA due to "promoter up" mutation are observed only when viral polymerase proteins are derived from avian influenza virus strains such as FPV/Bratislava used in the Hoffmann dissertation. In contrast, when viral polymerase proteins are derived from human influenza virus strains, the levels of transcription/replication achieved with the "promoter up" mutation-containing viral-like RNA are even lower than with the wild-type viral RNA, *i.e.*, this mutation has a "promoter down" effect. In other words, the Hoffmann dissertation discloses only the pol I-pol II system comprising mutant regulatory sequences, the use of which does not provide any expectation of success for the use of wild-type viral sequences required for successful production of infectious viruses. In sum, as one of ordinary skill in the art would readily observe, the artificiality of the system described in the Hoffmann thesis, while presenting a very interesting scientific story, does not provide a reasonable expectation of success with respect to expression of wild-type virus genes. Accordingly, the Hoffmann dissertation does not provide sufficient expectation of success to attempt the expression of all viral components in a cell using the pol I-pol II system, much less successfully achieving the difficult goal of reconstituting an infectious virus.

The Neumann *et al.* reference does not cure the deficiency of the Hoffmann dissertation, because it (i) does not teach the pol I-pol II system and (ii) discloses that transfection of the plasmids encoding vRNA should be accompanied by co-transfection of additional protein expression (mRNA) plasmids encoding viral polymerase complex (*i.e.*, plasmids encoding PB1, PB2, PA, nucleoproteins). Therefore, in contrast to the minimum plasmid-based system recited in the present claims (*e.g.*, encompassing 8 plasmids for 8-segmented influenza A virus), the total number of plasmids used in the method of Neumann *et*

al. exceeds the total number of gene segments from the source RNA virus (*e.g.*, encompasses 12-17 plasmids for an 8-segmented influenza A virus). Co-transfection of multiple plasmids described by Neumann *et al.* is cumbersome and technically complicated. Adding to that complexity the uncertainty of RNA and protein expression from a pol I-pol II system for wildtype viral proteins, and the level of uncertainty increases exponentially.

In contrast to the Examiner's assertion, the increased efficiency of the minimum plasmid-based system of the present invention is not merely a routine quantitative improvement over the system of Neumann *et al.*, but a major unexpected technical advance, which is likely to result in increased commercial success, *e.g.*, in improved vaccine production (*see* below). When explaining the advance made by the minimum pol I- pol II plasmid-based system of the present invention as compared to the plasmid-based system of Neumann *et al.*, the inventor and co-workers state in the Abstract of their *Virology* article (Hoffmann *et al.*, Virology, 2000, 267: 310-317):

> Recently, a system for the generation of influenza A virus entirely from cloned cDNAs was established (Neumann *et al.*, 1999, Proc. Natl. Acad. Sci. USA 96, 9345-9350)... Although this system is highly efficient in virus generation, the construction and cotransfection of 17 plasmids is cumbersome and may limit the use of this system to cell lines that can be transfected with high efficiencies. Synthesizing both vRNA and mRNA from one template would reduce the number of plasmids required for virus generation. Therefore, we generated a bidirectional transcription construct that contains cDNA encoding PB1 flanked by an RNA polymerase I (pol I) promoter for vRNA synthesis and an RNA polymerase II (pol II) promoter for mRNA synthesis... Because this approach reduces the number of plasmids required for virus generation, it also reduces the work necessary for cloning, probably enhances the efficiency of virus generation, and expands the use of the reverse-genetics system to cell lines for which efficient cotransfection of 17 plasmids cannot be achieved.

Furthermore, as correctly noted by the Examiner at p. 7 of the Office Action (second paragraph from the bottom), Neumann *et al.* teach that increasing the number of

different co-transfected protein expression (mRNA-encoding) plasmids from 4 to 9[3] increases the resulting viral titer (*see*, p. 9347 [¶ bridging left and right col.] and Table 1 at p. 9348; *see* also a comment at p. 5 of the instant specification). Based on this disclosure, in contrast to the Examiner's assertion, a person skilled in the art would be persuaded not to decrease but to increase the number of expression plasmids. That this is the case clearly follows from the Pekosz *et al.* Comment to Neumann *et al.* The authors of this Comment, Pekosz, He and Lamb, are leading scientists in the field, who are likely to have a much deeper understanding of the problem than "a person of ordinary skill in the art." However, in light of the data in Neumann *et al.*, even they suggest not to decrease but to increase the total number of the plasmids transfected. Thus, they state at p. 8806 (left col., ¶2): "The number of recombinant viruses rescued can be increased nearly 10-fold by including plasmids encoding the hemagglutinin, NA, M1, M2, and NS2 proteins under control of the pol II promoter in the transfection." Any suggestion to the contrary comes from the disclosure of the present application, not from the references.

Applicants also respectfully note that the Neumann *et al.* article teaches that the specific amount of each of the viral proteins is critical for successful production of the infectious virus. Specifically, it is stated at p. 9347 (left col., ¶3) that a reduced amount of the expression plasmid for production of PA protein should be used. Similarly, in the same year Gómez-Puertas *et al.* (J. Gen. Virol., 1999, 80: 1635-1645, attached as Exhibit H) reported that overexpression of influenza A NS2 protein from an expression plasmid has a negative effect on expression and overexpression of M2 and NS1 proteins has a negative effect on transmission of a reporter (CAT) RNA in cultured cells (COS-1 or MDCK). Based on these data, a skilled artisan would be discouraged from employing the pol I-pol II system disclosed in the Hoffmann dissertation for

[3] In the Action, the Examiner stated that this number is 8. According to the disclosure in Table 1 of Neumann *et al.* article, the total number of protein expression plasmids is 9.

in vivo reconstitution of an infectious virus, because the use of this system does not assume achieving unequal levels of expression proteins.

The Rejection Relies On Impermissible Hindsight

In view of the substantial reasons for unobviousness of the claimed invention set forth above, including that the combined references do not suggest, much less teach, the invention, that even if they suggest the invention there is no reasonable expectation of success, and indeed that the references themselves do not suggest the combination, it is clear that the rejection requires impermissible hindsight reconstruction of various unconnected bits and pieces of the references to sustain itself. The fact that, even after being in close collaboration with the present inventor, Erich Hoffmann, (as evidenced by co-authorship on most of the publications both several years before and after the filing date of the present application) and having great familiarity with his work (including the cited dissertation), Neumann *et al.* did not come up with the plasmid-based system recited in the present claims, clearly proves that the Examiner's reasoning reflects hindsight analysis and overlooks the tour de force represented by Hoffmann's success - the tour de force that was not predicted or predictable from the prior art references. It is well settled however, that such hindsight reconstruction is an error. The Examiner cannot rely on hindsight to arrive at a determination of obviousness. *Fritch*, 23 U.S.P.Q.2d at 1784. The Court of Appeals for the Federal Circuit has stated that "selective hindsight is no more applicable to the design of experiments than it is to the combination of prior art teachings. There must be a reason or suggestion in the art for selecting the procedure used, other than the knowledge learned from the Applicant's disclosure [*Interconnect Planning Corporation v. Fed.*, 227 USPQ 543, 551 (Fed. Cir., 1985)]". *Dow Chemical Co.*, 5 USPQ2d at 1532.

Commercial Success of the Invention

The non-obviousness of the minimum plasmid-based system recited in the present claims is further demonstrated by its projected commercial success[4] due to improved (*i.e.*, more efficient and rapid) manufacturing of anti-viral vaccines. For example, in a recently published article (Hoffmann *et al.*, Vaccine, 2002, 20: 3165-3170, Abstract attached as Exhibit I) the inventor and co-workers disclose the direct application of the minimum plasmid-based system of the present invention to rapid and reproducible generation of reassortant influenza A viruses. The authors produced high-yield 6+2 reassortants that had the antigenic determinants of the influenza virus strains A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), A/teal/HK/W312 (H6N1), and A/quail/HK/G1/97 (H9N2) and had a growth phenotype in embryonated chicken eggs which was equivalent to that of the wild-type virus.

In summary, neither the Hoffmann dissertation nor Neumann *et al.* provide any expectation of success or suggestion to be combined with the other reference or to modify the disclosed compositions, so that they in any way suggest the minimum pol I-pol II plasmid-based system for the generation of infectious negative strand RNA viruses from cloned viral cDNA and related methods encompassed by the present claims. It follows, that the rejected claims are not prima facie obvious over the cited art. Reconsideration and withdrawal of the obviousness rejection is believed to be in order.

---

[4] The Supreme Court in *Graham v. John Deere*, 383 U.S. 1, 148 USPQ 459 (1966), stated: "Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented. As indicia of obviousness or nonobviousness, these inquires may have relevancy..."

CONCLUSION

Applicants request entry of the foregoing amendments and remarks in the file history of this application. In view of the above amendments and remarks, it is respectfully submitted that claims 2-32, 39 and 42-45 are now in condition for allowance and such action is earnestly solicited. If the Examiner believes that a telephone conversation would help advance the prosecution in this case, the Examiner is respectfully requested to call the undersigned agent at (212) 527-7634. The Examiner is hereby authorized to charge any additional fees associated with this response to our Deposit Account No. 04-0100.

Respectfully submitted,

Dated: February 10, 2003

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Docket No.: 2427/1G772-US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Erich HOFFMANN

Serial No.:	09/844,517	Group Art Unit:	1648
Confirmation No.:	9063	Examiner:	Myron G. Hill
Filed:	April 27, 2001		

For: DNA TRANSFECTION SYSTEM FOR THE GENERATION OF INFECTIOUS INFLUENZA VIRUS

MARK-UP FOR AMENDMENT OF FEBRUARY 10, 2003

Pursuant to 37 C.F.R. §1.121, applicants provide the following mark-up copy of the amendments requested for claims in the above-referenced application. This document is submitted simultaneously with the Amendment and Response to the Office Action mailed on August 9, 2002.

AMENDMENT

CLAIMS:

1. Canceled

2. (Amended) The expression plasmid of claim [1] 42 wherein the pol I promoter is proximal to the polyadenylation signal and the pol I terminator sequence is proximal to the pol II promoter.

3. (Amended) The expression plasmid of claim [1] 42 wherein the pol I promoter is proximal to the pol II promoter and the pol I terminator sequence is proximal to the polyadenylation signal.

4. (Amended) The expression plasmid of claim [1] 42 wherein the plasmid [corresponds to a plasmid] has[ving] a map selected from the group consisting of pHW2000, pHW11 and pHW12.

5. (Amended) An [The] expression plasmid [of claim 1, further] comprising viral cDNA corresponding to a genomic segment of a segmented negative strand RNA virus, wherein the cDNA is inserted between [the] an RNA polymerase I (pol I) promoter and [the termination signal] a regulatory element for the synthesis of vRNA or cRNA with the exact 3' end, which are in turn inserted between an RNA polymerase II (pol II) promoter and a polyadenylation signal [viral gene segment].

6. (Unchanged) The expression plasmid of claim 5, wherein the negative strand RNA virus is a member of the *Orthomyxoviridae* virus family.

7. (Unchanged) The expression plasmid of claim 6, wherein the virus is an influenza A virus.

8. (Amended) The expression plasmid of claim 7, wherein the influenza viral gen[e]omic segment (i) encodes a [gene] protein selected from the group consisting of a viral polymerase complex protein, M protein, and NS protein; and [wherein the genes are] (ii) is derived from a strain well adapted to grow in cell culture or from an attenuated strain, or both.

9. (Unchanged) The expression plasmid of claim 6, wherein the virus is an influenza B virus.

10. (Unchanged) The expression plasmid of claim 8 wherein the plasmid has a map selected from the group consisting of pHW241-PB2, pHW242-PB1, pHW243-PA, pHW245-NP, pHW247-M, and pHW248-NS.

11. (Unchanged) The expression plasmid of claim 8 wherein the plasmid has a map selected from the group consisting of pHW181-PB2, pHW182-PB1, pHW183-PA, pHW185-NP, pHW187-M, and pHW188-NS.

12. (Amended) The expression plasmid of claim 7, wherein the influenza viral gen[e]omic segment [encodes] comprises a gene selected from the group consisting of a[n influenza] hemagglutinin (HA) gene and a neuraminidase (NA) gene.

13. (Unchanged) The expression plasmid of claim 12, wherein the influenza gene is from a pathogenic influenza virus strain.

14. (Unchanged) The expression plasmid of claim 12, wherein the plasmid has a map selected from the group consisting of pHW244-HA, pHW246-NA, pHW184-HA, and pHW186-NA.

15. (Amended) A minimum plasmid-based system for the generation of infectious segmented negative strand RNA viruses from cloned viral cDNA comprising a set of plasmids wherein each plasmid comprises one [autonomous] viral genomic segment, and wherein the viral cDNA corresponding to the [autonomous] viral genomic segment is inserted between an RNA polymerase I (pol I) promoter and [terminator sequences] a regulatory element for the synthesis of vRNA or cRNA with the exact 3' end, thereby resulting in expression of vRNA or cRNA, which are in turn inserted between an RNA polymerase II (pol II) promoter and a polyadenylation signal, thereby resulting in expression of viral mRNA.

16. (Amended) The minimum plasmid-based system of claim [15] 44, wherein the pol I promoter is proximal to the polyadenylation signal and the pol I terminator sequence is proximal to the pol II promoter.

17. (Amended) The minimum plasmid-based system of claim [15] 44, wherein the pol I promoter is proximal to the pol II promoter and the pol I terminator sequence is proximal to the polyadenylation signal.

18. (Amended) The minimum plasmid-based system of claim 15, wherein the negative strand RNA virus is a member of the *Orthomyxoviridae* virus family.

19. (Unchanged) The plasmid-based system of claim 18, wherein the virus is an influenza A virus.

20. (Unchanged) The plasmid-based system of claim 18, wherein the virus is an influenza B virus.

21. (Amended) The plasmid-based system of claim 19, wherein the viral gen[e]omic segment (i) encodes a protein selected from the group consisting of a viral polymerase complex protein, [an] M protein and [an] NS protein; and [wherein said genes are] (ii) is derived from a strain well adapted to grow in cell culture or from an attenuated strain, or both.

22. (Amended) The plasmid-based system of claim 19, wherein the viral genomic segment[s] comprises [genes which encode a protein selected from the group consisting of] hemagglutinin (HA) gene, [and] or neuraminidase (NA) gene, or both; wherein said genes are from a pathogenic influenza virus.

23. (Unchanged) The plasmid-based system of claim 19 wherein said system comprises one or more plasmids having a map selected from the group consisting of pHW241-PB2, pHW242-PB1, pHW243-PA, pHW244-HA, pHW245-NP, pHW246-NA, pHW247-M, and pHW248-NS.

24. (Unchanged) The plasmid-based system of claim 19, wherein said system comprises one or more plasmids having a map selected from the group consisting of pHW181-PB2, pHW182-PB1, pHW183-PA, pHW184-HA, pHW185-NP, pHW186-NA, pHW187-M, and pHW188-NS.

25. (Unchanged) A host cell comprising the plasmid-based system of claim 15.

26. (Unchanged) A host cell comprising the plasmid-based system of claim 18.

27. (Unchanged) A host cell comprising the plasmid-based system of claim 19.

28. (Unchanged) A host cell comprising the plasmid-based system of claim 22.

29. (Unchanged) A method for producing a negative strand RNA virus virion, which method comprises culturing the host cell of claim 25 under conditions that permit production of viral proteins and vRNA or cRNA.

30. (Unchanged) A method for producing an *Orthomyxoviridae* virion, which method comprises culturing the host cell of claim 26 under conditions that permit production of viral proteins and vRNA or cRNA.

31. (Unchanged) A method for producing an influenza virion, which method comprises culturing the host cell of claim 27 under conditions that permit production of viral proteins and vRNA or cRNA.

32. (Unchanged) A method for producing a pathogenic influenza virion, which method comprises culturing the host cell of claim 28 under conditions that permit production of viral proteins and vRNA or cRNA.

33-38. Canceled

39. (Unchanged) A method for generating an attenuated negative strand RNA virus, which method comprises:

(a) mutating one or more viral genes in the plasmid-based system of claim 15; and

(b) determining whether infectious RNA viruses produced by the system are attenuated.

40-41. Canceled

42. (New) The expression plasmid of claim 5, wherein the regulatory element for the synthesis of vRNA or cRNA with the exact 3' end is an RNA polymerase I (pol I) terminator sequence.

43. (New) The expression plasmid of claim 5, wherein the regulatory element for the synthesis of vRNA or cRNA with the exact 3' end is a ribozyme sequence.

44. (New) The minimum plasmid-based system of claim 15, wherein the regulatory element for the synthesis of vRNA or cRNA with the exact 3' end is an RNA polymerase I (pol I) terminator sequence.

45. (New) The minimum plasmid-based system of claim 15, wherein the regulatory element for the synthesis of vRNA or cRNA with the exact 3' end is a ribozyme sequence.

Respectfully submitted,

Dated: February 10, 2003

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APPENDIX

PENDING CLAIMS: February 10, 2003
(Application Serial No.: 09/844,517 Filed: April 27, 2001)

2. (Amended) The expression plasmid of claim 42 wherein the pol I promoter is proximal to the polyadenylation signal and the pol I terminator sequence is proximal to the pol II promoter.

3. (Amended) The expression plasmid of claim 42 wherein the pol I promoter is proximal to the pol II promoter and the pol I terminator sequence is proximal to the polyadenylation signal.

4. (Amended) The expression plasmid of claim 42 wherein the plasmid has a map selected from the group consisting of pHW2000, pHW11 and pHW12.

5. (Amended) An expression plasmid comprising viral cDNA corresponding to a genomic segment of a segmented negative strand RNA virus, wherein the cDNA is inserted between an RNA polymerase I (pol I) promoter and a regulatory element for the synthesis of vRNA or cRNA with the exact 3' end, which are in turn inserted between an RNA polymerase II (pol II) promoter and a polyadenylation signal.

6. (Unchanged) The expression plasmid of claim 5, wherein the negative strand RNA virus is a member of the *Orthomyxoviridae* virus family.

7. (Unchanged) The expression plasmid of claim 6, wherein the virus is an influenza A virus.

8. (Amended) The expression plasmid of claim 7, wherein the influenza viral genomic segment (i) encodes a protein selected from the group consisting of a viral polymerase complex protein, M protein, and NS protein; and (ii) is derived from a strain well adapted to grow in cell culture or from an attenuated strain, or both.

9. (Unchanged) The expression plasmid of claim 6, wherein the virus is an influenza B virus.

PENDING CLAIMS: February 10, 2003
(Application Serial No.: 09/844,517 Filed: April 27, 2001)

10. (Unchanged) The expression plasmid of claim 8 wherein the plasmid has a map selected from the group consisting of pHW241-PB2, pHW242-PB1, pHW243-PA, pHW245-NP, pHW247-M, and pHW248-NS.

11. (Unchanged) The expression plasmid of claim 8 wherein the plasmid has a map selected from the group consisting of pHW181-PB2, pHW182-PB1, pHW183-PA, pHW185-NP, pHW187-M, and pHW188-NS.

12. (Amended) The expression plasmid of claim 7, wherein the influenza viral genomic segment comprises a gene selected from the group consisting of a hemagglutinin (HA) gene and a neuraminidase (NA) gene.

13. (Unchanged) The expression plasmid of claim 12, wherein the influenza gene is from a pathogenic influenza virus strain.

14. (Unchanged) The expression plasmid of claim 12, wherein the plasmid has a map selected from the group consisting of pHW244-HA, pHW246-NA, pHW184-HA, and pHW186-NA.

15. (Amended) A minimum plasmid-based system for the generation of infectious segmented negative strand RNA viruses from cloned viral cDNA comprising a set of plasmids wherein each plasmid comprises one viral genomic segment, and wherein the viral cDNA corresponding to the viral genomic segment is inserted between an RNA polymerase I (pol I) promoter and a regulatory element for the synthesis of vRNA or cRNA with the exact 3' end, thereby resulting in expression of vRNA or cRNA, which are in turn inserted between an RNA polymerase II (pol II) promoter and a polyadenylation signal, thereby resulting in expression of viral mRNA.

PENDING CLAIMS: February 10, 2003
(Application Serial No.: 09/844,517 Filed: April 27, 2001)

16. (Amended) The minimum plasmid-based system of claim 44, wherein the pol I promoter is proximal to the polyadenylation signal and the pol I terminator sequence is proximal to the pol II promoter.

17. (Amended) The minimum plasmid-based system of claim 44, wherein the pol I promoter is proximal to the pol II promoter and the pol I terminator sequence is proximal to the polyadenylation signal.

18. (Amended) The minimum plasmid-based system of claim 15, wherein the negative strand RNA virus is a member of the *Orthomyxoviridae* virus family.

19. (Unchanged) The plasmid-based system of claim 18, wherein the virus is an influenza A virus.

20. (Unchanged) The plasmid-based system of claim 18, wherein the virus is an influenza B virus.

21. (Amended) The plasmid-based system of claim 19, wherein the viral genomic segment (i) encodes a protein selected from the group consisting of a viral polymerase complex protein, M protein and NS protein; and (ii) is derived from a strain well adapted to grow in cell culture or from an attenuated strain, or both.

22. (Amended) The plasmid-based system of claim 19, wherein the viral genomic segment comprises hemagglutinin (HA) gene, or neuraminidase (NA) gene, or both; wherein said genes are from a pathogenic influenza virus.

23. (Unchanged) The plasmid-based system of claim 19 wherein said system comprises one or more plasmids having a map selected from the group consisting of pHW241-PB2, pHW242-PB1, pHW243-PA, pHW244-HA, pHW245-NP, pHW246-NA, pHW247-M, and pHW248-NS.

24. (Unchanged) The plasmid-based system of claim 19, wherein said system comprises one or more plasmids having a map selected from the group consisting of pHW181-PB2, pHW182-PB1, pHW183-PA, pHW184-HA, pHW185-NP, pHW186-NA, pHW187-M, and pHW188-NS.

25. (Unchanged) A host cell comprising the plasmid-based system of claim 15.

26. (Unchanged) A host cell comprising the plasmid-based system of claim 18.

27. (Unchanged) A host cell comprising the plasmid-based system of claim 19.

28. (Unchanged) A host cell comprising the plasmid-based system of claim 22.

29. (Unchanged) A method for producing a negative strand RNA virus virion, which method comprises culturing the host cell of claim 25 under conditions that permit production of viral proteins and vRNA or cRNA.

30. (Unchanged) A method for producing an *Orthomyxoviridae* virion, which method comprises culturing the host cell of claim 26 under conditions that permit production of viral proteins and vRNA or cRNA.

31. (Unchanged) A method for producing an influenza virion, which method comprises culturing the host cell of claim 27 under conditions that permit production of viral proteins and vRNA or cRNA.

32. (Unchanged) A method for producing a pathogenic influenza virion, which method comprises culturing the host cell of claim 28 under conditions that permit production of viral proteins and vRNA or cRNA.

APPENDIX

PENDING CLAIMS: February 10, 2003
(Application Serial No.: 09/844,517 Filed: April 27, 2001)

39. (Unchanged) A method for generating an attenuated negative strand RNA virus, which method comprises:

(a) mutating one or more viral genes in the plasmid-based system of claim 15; and

(b) determining whether infectious RNA viruses produced by the system are attenuated.

42. (New) The expression plasmid of claim 5, wherein the regulatory element for the synthesis of vRNA or cRNA with the exact 3' end is an RNA polymerase I (pol I) terminator sequence.

43. (New) The expression plasmid of claim 5, wherein the regulatory element for the synthesis of vRNA or cRNA with the exact 3' end is a ribozyme sequence.

44. (New) The minimum plasmid-based system of claim 15, wherein the regulatory element for the synthesis of vRNA or cRNA with the exact 3' end is an RNA polymerase I (pol I) terminator sequence.

45. (New) The minimum plasmid-based system of claim 15, wherein the regulatory element for the synthesis of vRNA or cRNA with the exact 3' end is a ribozyme sequence.

09/644,517

#18 attach Exhibits

LIST OF EXHIBITS:

A. Hoffmann *et al.*, Proc. Natl. Acad. Sci. USA, 2002, 99: 11411-6

B. Jackson *et al.*, J. Virol., 2002, 76: 11744-7

C. Wagner *et al.*, 2001, J. Virol., 75: 9282-6

D. Flick and Pettersson, J. Virol., 2001, 75: 1643-55

E. Flick *et al.*, J. Virol., 2002, 76: 10849-60

F. Pekosz *et al.*, Proc. Natl. Acad. Sci. USA, 1999, 96: 8804-6

G. Crescenzo-Chaigne *et al.*, Virology, 2002, 303: 240-252

H. Gómez-Puertas *et al.*, J. Gen. Virol., 1999, 80: 1635-45

I. Hoffmann *et al.*, Vaccine, 2002, 20: 3165-70, Abstract

Rescue of influenza B virus from eight plasmids

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Contributed by Robert G. Webster, July 3, 2002

Influenza B virus causes a significant amount of morbidity and mortality, yet the systems to produce high yield inactivated vaccines for these viruses have lagged behind the development of those for influenza A virus. We have established a plasmid-only reverse genetics system for the generation of recombinant influenza B virus that facilitates the generation of vaccine viruses without the need for time consuming coinfection and selection procedures currently required to produce reassortants. We cloned the eight viral cDNAs of influenza B/Yamanashi/166/98, which yields relatively high titers in embryonated chicken eggs, between RNA polymerase I and RNA polymerase II transcription units. Virus was detected as early as 3 days after transfection of cocultured COS7 and Madin–Darby canine kidney cells and achieved levels of 10^6-10^7 plaque-forming units per ml of cell supernatant 6 days after transfection. The full-length sequence of the recombinant virus after passage into embryonated chicken eggs was identical to that of the input plasmids. To improve the utility of the eight-plasmid system for generating 6 + 2 reassortants from recently circulating influenza B strains, we optimized the reverse transcriptase–PCR for cloning of the hemagglutinin (HA) and neuraminidase (NA) segments. The six internal genes of B/Yamanashi/166/98 were used as the backbone to generate 6 + 2 reassortants including the HA and NA gene segments from B/Victoria/504/2000, B/Hong Kong/330/2001, and B/Hawaii/10/2001. Our results demonstrate that the eight-plasmid system can be used for the generation of high yields of influenza B virus vaccines expressing current HA and NA glycoproteins from either of the two lineages of influenza B virus.

Influenza is a major cause of morbidity and mortality worldwide. In the USA alone, it is estimated that influenza is responsible for approximately 20,000 deaths each year (1). The morbidity associated with these epidemics is caused by both types of influenza virus, influenza A and influenza B. The immune response elicited by infection with a specific influenza strain is long lasting and protects the individual from experiencing illness on subsequent exposures to other strains that are antigenically similar. However, immunity conferred by infection with a specific influenza strain does not confer protection to other influenza types, subtypes, or antigenically divergent strains of the same subtype. Vaccines to prevent influenza generally contain the surface hemagglutinin (HA) and neuraminidase (NA) glycoproteins from the two currently circulating influenza A subtypes (i.e., H3N2 and H1N1) and one circulating influenza B strain. Because of the frequent emergence of new antigenic variants created by antigenic shift or antigenic drift, vaccines have to be updated frequently.

Currently, two major lineages of influenza B viruses are circulating in humans: the B/Yamagata/16/88-like and B/Victoria/2/87-like strains. Although the B/Yamagata/16/88-like strains have been the predominant strains circulating for the past 10 years, these two lineages circulate concurrently and both have been responsible for annual influenza epidemics (2–4). Antigenic drift is responsible for the divergence of influenza B surface antigens. Antigenic drift is caused by amino acid changes in HA and NA, which are caused by nucleotide misincorporation during viral replication. In addition, insertion and/or deletion of nucleotides in the HA and NA gene segments of influenza B viruses have been shown to be a source of antigenic diversity and evolution (5, 6). Although it is well established that influenza B viruses can evolve by reassortment (6, 7), little is known about the animal reservoir for this virus. Recently, influenza B viruses have been isolated from seals, demonstrating that influenza B viruses are not restricted to humans and raising concerns about the potential for influenza B viruses to emerge with new antigenic properties (8).

For optimal effectiveness, influenza vaccines must contain antigens that are similar to those of the currently circulating strains. The simplest approach to produce an inactivated vaccine is to identify a wild-type (wt) strain that has appropriate antigenic characteristics and grows well in eggs. However, this approach depends on the availability of a high yield wild-type isolate. A second method, classical reassortment, requires coinfection of two viruses, one expressing the desirable HA and/or NA and the other a vaccine strain that has the appropriate biological characteristics. The desired reassortant is identified among the progeny and purified. For inactivated influenza A vaccine production, the vaccine strain A/PR/8/34, which expresses the desirable characteristics of high yield in eggs, is coinfected with a virus expressing the current antigens, and a high growth reassortant is isolated (9). For live, attenuated influenza A vaccines, the wild-type HA and NA gene segments can be reassorted onto the live, attenuated ca A/Ann Arbor/6/60 background to produce attenuated vaccine strains (10). These reassorted vaccines have been shown to be safe and effective for the prevention of influenza (11). Currently, although live, attenuated influenza B vaccine strains can be produced by classical reassortment, there are no influenza B strains used for classical reassortment for production of inactivated vaccines. There are at least two limitations to producing these reassortants. First, influenza A/PR/8/34 cannot be used to reassort with influenza B HA and NA gene segments. Influenza A and B viruses have evolved separately, and reassortment between these two types has never been observed either in nature or in tissue culture (12, 13). An influenza B vaccine strain with high yield growth properties in eggs, analogous to A/PR/8/34, for production of inactivated vaccine has not been reported, and the procedures to produce 6 + 2 reassortants in the laboratory have been more difficult than for influenza A strains. Second, recent advances in reverse genetics of influenza A viruses may enable more efficient methods to generate recombinant vaccine strains expressing wt HA and NA from a vaccine strain with the appropriate biological characteristics (14–16). Generation of infectious virus directly from plasmid clones has enabled several different influenza A HA and NA subtypes to be expressed from the A/PR/8/34 backbone without the need for coinfection and extensive selection of the progeny (17, 18).

Despite one recent report on the generation of virus like particles (VLP) for influenza B virus from plasmids (19), the development of a plasmid-only system for generation of recombinant, infectious influenza B virus poses major challenges. To date, no plasmid-based system for influenza B virus rescue has been reported. Obviously, the genomic sequence (14642 nucleotides), including the entire noncoding and coding regions of all eight gene segments, has to be cloned into plasmids and sequenced. Because the viral

MICROBIOLOGY

Abbreviations: HA, hemagglutinin; MDCK, Madin–Darby canine kidney; NCR, noncoding region; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural; pol I, RNA polymerase I; pol II, RNA polymerase II; pfu, plaque-forming unit; RT, reverse transcriptase; vRNA, viral RNA; wt, wild type; $TCID_{50}$, tissue culture 50% infective dose.

†To whom reprint requests should be addressed. E-mail: hoffmane@medimmune.com.

Table 1. Primer set used for RT-PCR amplification of the eight vRNAs of B/Yamanashi/166/98

Gene	Forward primer	Reverse primer
PB1 [1A]	**Bm-PB1b-1:** TATTCGTCTCAGGGAGCAGAAGCGGAGCCTTTAAGATG	**Bm-PB1b-1200R:** TATTCGTCTCGATGCCGTTCCTTCTTCATTGAAGAATGG
PB1 [1B]	**Bm-PB1b-1220:** TATTCGTCTCGGCATCTTTGTCGCCTGGGATGATGATG	**Bm-PB1b-2369R:** ATATCGTCTCGTATTAGTAGAAACACGAGCCTT
PB2 [2A]	**Bm-PB2b-1:** TATTCGTCTCAGGGAGCAGAAGCGGAGCGTTTTCAAGATG	**Bm-PB2b-1145R:** TATTCGTCTCTCTCATTTTGCTCTTTTTTAATATTCCCC
PB2 [2B]	**Bm-PB2b-1142:** TATTCGTCTCATGAGAATGGAAAAACTACTAATAAATTCAGC	**Bm-PB2b-2396R** ATATCGTCTCGTATTAGTAGAAACACGAGCATT
PA [3A]	**Bm-PAb-1:** TATTCGTCTCAGGGAGCAGAAGCGGTGCGTTTGA	**Bm-PAb-1261R:** TATTCGTCTCCCAGGGCCCTTTTACTTGTCAGAGTGC
PA [3B]	**Bm-PAb-1283:** TATTCGTCTCTCCTGGATCTACCAGAAATAGGGCCAGAC	**Bm-PAb-2308R:** ATATCGTCTCGTATTAGTAGAAACACGTGCATT
HA	**MDV-B 5′ BsmBI-HA:** TATTCGTCTCAGGGAGCAGAAGCAGAGCATTTTCTAATATC	**MDV-B 3′ BsmBI-HA:** ATATCGTCTCGTATTAGTAGTAACAAGAGCATTTTTC
NP	**MDV-B 5′ BsmBI-NP:** TATTCGTCTCAGGGAGCAGAAGCACAGCATTTTCTTGTG	**MDV-B 3′ BsmBI-NP:** ATATCGTCTCGTATTAGTAGAAACAACAGCATTTTTTAC
NA	**Bm-NAb-1:** TATTCGTCTCAGGGAGCAGAAGCAGAGCA	**Bm-NAb-1557R:** ATATCGTCTCGTATTAGTAGTAACAAGAGCATTTT
M	**MDV-B 5′ BsmBI-M:** TATTCGTCTCAGGGAGCAGAAGCACGCACTTTCTTAAAATG	**MDV-B 3′ BsmBI-M:** ATATCGTCTCGTATTAGTAGAAACAACGCACTTTTTCCAG
NS	**MDV-B 5′ BsmBI-NS:** TATTCGTCTCAGGGAGCAGAAGCAGAGGATTTGTTTAGTC	**MDV-B 3′ BsmBI-NS:** ATATCGTCTCGTATTAGTAGTAACAAGAGGATTTTTAT

The sequences complementary to the influenza sequences are shown in bold. The 5′-ends have recognition sequences for the restriction endonuclease *Bsm*BI (Bm, CGTCTCN1/N5). The design of the primers for PB1, PB2, and PA allowed the amplification of two fragments (1A, 1B, 2A, 2B, 3A, 3B).

sequences in the plasmids possibly represent nonviable variants of the virus population or have mutations introduced by reverse transcriptase (RT)-PCR, multiple plasmids for each segment have to be characterized. A single mutation in one of the plasmids could result in failure to rescue virus. The established plasmid-only systems for influenza A virus are based on the *in vivo* transcription of viral RNA (vRNA) or cRNA from an RNA polymerase I (pol I) promoter on the plasmid (20, 21). These 5′ and 3′ termini of vRNAs or cRNAs are different between type A and type B virus and contain sequences that are unique for each of the eight segments. However, most sequences deposited in GenBank for influenza B virus do not include those end sequences. In addition, some of the published terminal regions differ between isolates either because the viruses have different sequences and/or because of sequencing errors.

The proteins of type A and type B viruses with analogous functions show homologies ranging from about 35% for nucleoprotein (NP) to 60% for the polymerase subunit PB1, the most conserved protein among influenza viruses (22, 23). In addition to sequence differences between influenza A and B virus, the genetic organization of these two viruses is different. RNA segment 6 of influenza A virus is monocistronic, coding for the NA protein, whereas segment 6 of influenza B virus encodes NA and NB proteins by using a bicistronic mRNA with two overlapping ORFs. The NB protein of influenza B virus is a membrane protein that is believed to serve a function similar to that of the M2 protein of influenza A viruses (24–26). Furthermore, although RNA segment 7 of both influenza A and B viruses encodes the matrix protein M1, the organizations of their respective M2 genes are quite different. The M2 protein of influenza A virus is translated from an mRNA generated by splicing (27, 28). RNA segment 7 of influenza B viruses is bicistronic, containing two ORFs characterized by overlapping of the termination codon of the M1 gene and the initiation codon of the BM2 gene (29). The BM2 protein is a phosphoprotein that is synthesized late in the phase of infection and incorporated into virions (30). Influenza A viruses have no counterpart for the BM2 protein. Recently, a second ORF in the PB1 gene of influenza A virus, termed PB1-F2, was reported; influenza B viruses do not have this ORF (31). The sequence and organizational differences between type A and type B viruses combined with uncertainties of vRNA stability and integrity do not allow one to predict the success of influenza B virus rescue. The utility of a reverse genetics system for generation of influenza B virus can be tested only by plasmid-driven expression of all influenza B virus genes and evaluation of growth conditions in suitable host cells for virus rescue.

Here, we report the establishment of an eight-plasmid system that enables the generation of infectious, recombinant influenza B vaccine strains, and provides a system that will enable the genetic characterization of influenza B viral genes. Our results demonstrate that the eight-plasmid system allows reproducible and rapid generation of B/Yamanashi/166/98 and 6 + 2 reassortants derived from influenza B strains circulating in humans. This system can be applied to the rapid generation of influenza B virus vaccines expressing current antigens.

Materials and Methods

Virus Strains. The virus strain B/Yamanashi/166/98 was obtained from the repository of St. Jude Children's Research Hospital (Memphis, TN). B/Victoria/504/2000, B/Hong Kong/330/2001, and B/Hawaii/10/2001 viruses were provided by N. Cox and A. Klimov (Centers for Disease Control, Atlanta, GA).

RT-PCR. The RNeasy Kit (Qiagen, Chatsworth, CA) was used to extract vRNA from 100 μl of allantoic fluid from infected embryonated chicken eggs. The RNA was eluted into 40 μl of H_2O. For RT-PCR of the eight segments, the One Step RT-PCR kit (Qiagen) was used according to the protocol provided. One microliter of RNA was used for each reaction. The RT reaction was performed for 50 min at 50°C, followed by 15 min at 94°C. The PCR reaction was carried out by 25 cycles with the following conditions: 94°C for 30 s, 54°C for 30 s, and 72°C for 3 min. The primers used for construction of plasmids representing the eight segments of B/Yamanashi/166/98 are shown in Table 1. The PB1, PB2, and PA genes were amplified by using segment-specific primers with *Bsm*BI sites that allow the generation of two fragments. The primers with nucleotides complementary to the internal regions of these genes have *Bsm*BI sites facilitating the exact fusion of the two amplified fragments in a three-fragment ligation reaction with the *Bsm*BI-digested vector pAD3000.

For RT-PCR amplification of the HA and the NA segments, we

used the primer pair Bm-NAb-1/Bm-NAb-1557R (Table 1), which allowed the amplification of both segments under the same RT-PCR conditions as described above. The PCR products were sequenced with internal segment-specific primers to derive a consensus sequence. For amplification of the PA gene of wt and recombinant B/Yamanashi/166/98, the forward primer eh153s-1 (ATG AAA AGC GAA AAA GCT AAC GAAA ATT TCC) and reverse primer eh253r-2 (GTT ACT AAT ACA TTC TTG TAT TCC AGA ATA CA) were used. The sequence shown in Fig. 3 was obtained by sequencing the PCR product with the forward primer eh153s-1. The sequence of template DNA was determined by using Rhodamine or dRhodamine dye-terminator cycle sequencing ready reaction kits with AmpliTaq DNA polymerase FS (Perkin–Elmer). Samples were separated by electrophoresis and analyzed on PE/ABI model 373, model 373 Stretch, or model 377 DNA sequencers.

Cloning of Plasmids. The cloning vector pAD3000 was derived from pHW2000 (16) by replacing the bovine GH polyadenylation signal with the SV40 late mRNA polyadenylation signal. RT-PCR fragments were isolated, digested with *Bsm*BI, and inserted into the *Bsm*BI-digested plasmid pAD3000.

To ensure that the plasmids represent wt viral sequences, a consensus sequence for all eight segments of B/Yamanashi/166/98 was constructed from sequence data in GenBank for the coding regions PB1 (AF102007), PB2 (AF101990), PA (AF102024), HA (AF100355), NP (AF100373), M (AF100392), and nonstructural (NS) (AF100410) (7, 32), by direct sequencing of the RT-PCR product of segment 6, and by sequencing the noncoding regions (NCRs) of the viral RNAs. Two sets of eight plasmids derived from B/Yamanashi/166/98 were constructed independently; some of the plasmids had mutations resulting in amino acid changes. The changes were corrected to reflect the consensus sequence by site-directed mutagenesis using PfuTurbo DNA polymerase (Stratagene) or exchanged by cloning with other unaltered plasmid sequences. The resultant plasmids were sequenced and designated pAB251-PB1, pAB252-PB2, pAB253-PA, pAB254-HA, pAB255-NP, pAB256-NA, pAB257-M, and pAB258-NS. pAB253-PA differs in three nucleotides from the consensus at the following positions: 1280A → 1280G, 1283T → 1283C, and 1289C → 1289T. These substitutions served as a genetic tag for the recombinant virus and were introduced by using the primer Bm-PAb-1261R (Table 1). These nucleotide differences are silent, resulting in no change in the amino acid sequence of the PA protein.

Transfection and Generation of Virus. The protocol used for the rescue of influenza B virus was based on the protocol for generation of influenza A virus (16). Briefly, COS7 cells were transiently cocultured with Madin–Darby canine kidney (MDCK) cells in six-well plates. Two microliters of TransIT-LT-1 (Mirrus, Madison, WI) per 1 μg of DNA was mixed, incubated at room temperature for 45 min, and added to the cells. After 6–15 h, the DNA-transfection mixture was replaced by Opti-MEM I (Life Technologies, Rockville, MD). The cells were incubated at 33°C. To ensure that the generated recombinant viruses were able to replicate (cleavage of the HA to produce infectious virus) 2, 4, and 6 days after transfection, 1 ml of Opti-MEM I containing 1 μg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-trypsin was added to the cells.

For plaque assay, supernatants were titrated on MDCK cells, which were incubated with an 0.8% agarose overlay for 3 days at 33°C. For infection of eggs, the supernatants of transfected cells were harvested 6 or 7 days after transfection, and 100 μl of the virus dilutions in Opti-MEM I were injected into 11-day-old embryonated chicken eggs at 33°C. The titer was determined 3 days after inoculation by $TCID_{50}$ (tissue culture 50% infective dose) assay in MDCK cells.

Results

Generation of B/Yamanashi/166/98 from Eight Plasmids. To construct eight plasmids representing the virus strain B/Yamanashi/166/98, viral RNA was reverse transcribed and amplified by PCR. The resultant cDNA fragments were inserted into pAD3000, which contains a pol I transcription unit designed to synthesize vRNA from one strand, flanked by an RNA polymerase II (pol II) transcription unit to synthesize mRNA from the opposite strand. All viral RNAs and mRNAs/proteins should be produced intracellularly after transfection of a primate cell (Fig. 1). The viral cDNAs cloned into the resultant plasmids were sequenced in their entirety and represent the consensus sequence of the eight segments of B/Yamanashi/166/98. These plasmids were designated pAB251-PB1, pAB252-PB2, pAB253-PA, pAB254-HA, pAB255-NP, pAB256-NA, pAB257-M, and pAB258-NS.

To test whether infectious B/Yamanashi/166/98 virus could be generated from the constructed eight plasmids, cocultured COS7-MDCK cells were transfected with 1 μg of each plasmid. Characteristic virus induced cytopathic effect (CPE) was evident in the MDCK cells 5 to 7 days posttransfection and required the transfection of all eight plasmids to produce CPE (Table 2). In addition, the supernatants from these transfected cells were plated out at a variety of dilutions on fresh monolayers of MDCK cells, and infectious virus was detected only when all eight plasmids were present. These results indicated that infectious virus was generated from cloned cDNAs.

To determine the efficiency of the DNA transfection system for virus generation, supernatants of transfected COS7-MDCK cells or COS7 cells alone were titrated at day 2, 3, 4, 5, and 6 after transfection on fresh MDCK cells. No virus could be detected 2 days after transfection. Three days after transfection, 2×10^3 plaque-forming units (pfu)/ml of virus were detected in the supernatant of transfected COS7-MDCK cells, and 1×10^1 pfu/ml were detected on COS7 cells alone (Fig. 2). This yield increased in COS7-MDCK cells to 5×10^7 pfu/ml and in COS7 cells alone to 2×10^3 pfu/ml 6 days posttransfection. No virus was detected in supernatants derived from cells transfected with only seven plasmids.

To formally prove that the virus in the supernatants of infected cells was derived from the input plasmids, the presence of the three silent nucleotide differences in PA were identified. RT-PCR was performed with allantoic fluid from embryonated chicken eggs infected with either wt B/Yamanashi/166/98 (wtB-Yam) or supernatant from cells transfected with eight plasmids (recB-Yam). The region of the PA gene encompassing nucleotides 1280–1290 was amplified by RT-PCR and sequenced (Fig. 3). Sequencing of the PCR products revealed that recB-Yam contained the three expected nucleotide changes relative to wtB-Yam. Thus, the recombinant virus was derived from the input plasmids.

In addition, all eight gene segments of recB-Yam were amplified by RT-PCR to evaluate the fidelity of the *in vivo* transcription of the type B segments. The sequences of the PCR products were determined by sequencing using segment-specific primers. There were no nucleotide differences between the sequences of the plasmid-derived, recombinant virus and those of the plasmids used for transfection. These data show that, after transfection of eight plasmids, all viral cDNAs are transcribed with high fidelity intracellularly, resulting in reproducible and efficient *de novo* generation of the influenza virus B/Yamanashi/166/98.

RT-PCR and Cloning of Genes Representing the HA and NA of Influenza B Virus. In general, for use of the plasmid-based system for rescue of vaccine virus strains, it would be advantageous to have a simple and reliable method for sequence determination and cloning of the genes representing the HA and NA glycoproteins. The primers were optimized for simultaneous RT-PCR amplification of the HA and NA segments. Comparison of the terminal regions of the vRNA

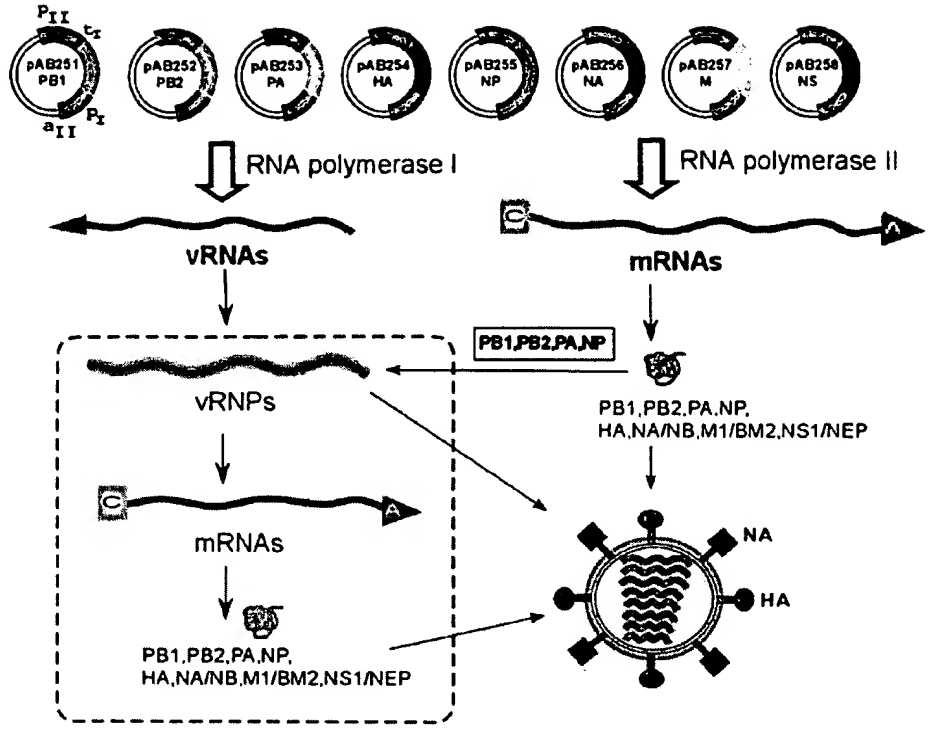

Fig. 1. Eight-plasmid bidirectional transcription system for the generation of influenza B virus. The eight plasmids contain cDNAs representing the eight gene segments of influenza B virus. In each of the plasmids, the cDNAs are flanked by pol I (p_I) promoter/terminator (t_I) sequences. The RNA pol I transcription unit is flanked by pol II promoter (p_{II})/polyadenylation (a_{II}) sequences. After transfection of these constructs into COS7 cells, cellular pol I synthesize virus-like noncapped negative sense vRNAs and pol II capped mRNAs encoding the viral proteins. After translation of PB1, PB2, PA, and NP proteins, the eight negative sense vRNAs are transcribed and replicated. Ultimately, the viral ribonucleoproteins (vRNPs) and structural proteins derived from cellular transcription or viral amplification (dashed rectangle) are assembled into new virus particles.

representing the NCR of segment 4 (HA) and segment 6 (NB/NA) revealed that the 20 terminal nucleotides at the 5′ end and 15 nucleotides at the 3′ end were identical between the HA and NA genes of influenza B viruses. A primer pair for RT-PCR [(underlined sequences are influenza B virus specific) Bm-NAb-1, TAT TCG TCT CAG GGA GCA GAA GCA GAG CA; Bm-NAb-1557R, ATA TCG TCT CGT ATT AGT AGT AAC AAG AGC ATT TT] was synthesized and used to simultaneously amplify the HA and NA genes from various influenza B strains (Fig. 4). The HA and NA PCR fragments of B/Victoria/504/2000, B/Hawaii/10/2001, and B/Hong Kong/330/2001 were isolated, digested with *Bsm*BI, and inserted into pAD3000 (Table 2). These results demonstrated the applicability of these primers for the efficient generation of plasmids containing the influenza B HA and NA genes from several different wt viruses. The RT-PCR products can be used for sequencing and/or cloning into the expression plasmids.

Previously, a similar approach was used for the amplification of all eight segments of influenza A virus (33).

Generation of 6 + 2 Reassortants with B/Yamanashi/166/98 as Backbone. Influenza B viruses segregate between two distinct lineages, B/Victoria/2/87-like viruses and B/Yamagata/16/88-like viruses (2–6). To demonstrate the utility of B/Yamanashi/166/98 (a B/Yamagata/16/88-like virus) to efficiently express antigens from both these lineages, reassortants containing PB1, PB2, PA, NP, M, NS from B/Yamanashi/166/98, and the HA and NA from strains representing both the Victoria and Yamagata lineages (6 + 2 reassortants) were generated.

Transiently cocultured COS7-MDCK cells were cotransfected with six plasmids representing B/Yamanashi/166/98 and two plasmids containing the cDNA of the HA and NA segments of two strains from the B/Victoria/2/87 lineage, B/Hong Kong/330/2001

Table 2. Plasmid set used for the generation of B/Yamanashi/166/98 and 6 + 2 reassortants

Segment		Plasmid set			
1	—	pAB251-PB1	pAB251-PB1	pAB251-PB1	pAB251-PB1
2	pAB252-PB2	pAB252-PB2	pAB252-PB2	pAB252-PB2	pAB252-PB2
3	pAB253-PA	pAB253-PA	pAB253-PA	pAB253-PA	pAB253-PA
4	pAB254-HA	pAB254-HA	**pAB281-HA**	**pAB285-HA**	**pAB287-HA**
5	pAB255-NP	pAB255-NP	pAB255-NP	pAB255-NP	pAB255-NP
6	pAB256-NA	pAB256-NA	**pAB291-NA**	**pAB295-NA**	**pAB297-NA**
7	pAB257-M	pAB257-M	pAB257-M	pAB257-M	pAB257-M
8	pAB258-NA	pAB258-NA	pAB258-NA	pAB258-NA	pAB258-NA
Recombinant virus		8 B/Yamanashi/166/98	6 + 2 B/Victoria/504/2000	6 + 2 B/Hawaii/10/2001	6 + 2 B/Hong Kong/330/2001
pfu/ml*	0	4×10^6	9×10^6	6×10^6	7×10^6

*Supernatants of cocultured COS7-MDCK cells were titrated 6 or 7 days after transfection, and the viral titer was determined by plaque assays on MDCK cells. Plasmids containing the HA or NA cDNA of currently circulating strains are shown in bold.

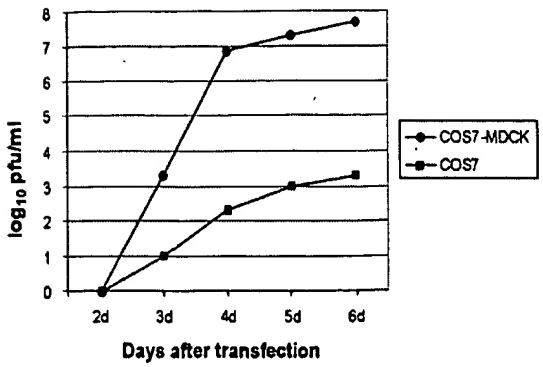

Fig. 2. Kinetics of virus generation after transfection. COS7 or cocultured COS7-MDCK cells were transfected with eight plasmids representing the eight segments of B/Yamanashi/166/98. The virus yield of the supernatant was determined at different times after transfection by plaque assay on MDCK cells.

and B/Hawaii/10/2001, and one strain from the B/Yamagata/16/88 lineage, B/Victoria/504/2000. Six to seven days after transfection, the supernatants were titrated on fresh MDCK cells. All three 6 + 2 reassortant viruses had titers between 4–9 × 10^6 pfu/ml (Table 2). These data demonstrated that the six internal genes of B/Yamanashi/166/98 could efficiently form infectious virus with HA and NA gene segments from both influenza B lineages.

Relatively high titers are obtained by replication of wt B/Yamanashi/166/98 in eggs. Experiments were performed to determine whether this property was an inherent phenotype of the six "internal" genes of this virus. To evaluate this property, the yield of WT B/Victoria/504/2000, which replicated only moderately in eggs, was compared with the yield of the 6 + 2 reassortant expressing the B/Victoria/504/2000 HA and NA. These viruses in addition to wt and recombinant B/Yamanashi/166/98 were each inoculated into three or four embryonated chicken eggs, at either 100 or 1,000 pfu. Three days after infection, the allantoic fluids were harvested from the eggs and the $TCID_{50}$ titers were determined on MDCK cells. The 6 + 2 reassortants produced similar quantities of virus in the allantoic fluid to the wt and recombinant B/Yamanashi/166/98 strain (Fig. 5). The difference in titer between B/Victoria/504/2000 and the 6 + 2 recombinant was ≈1.6 $\log_{10}$ $TCID_{50}$ (0.7–2.5 $\log_{10}$ $TCID_{50}$/ml, 95% confidence interval). The difference between B/Victoria/504/2000 and the 6 + 2 recombinant was confirmed on three separate experiments ($P < 0.001$). These results demonstrated that the egg growth properties of B/Yamanashi/166/98 could be conferred to HA and NA antigens that are normally expressed from strains that replicated poorly in eggs.

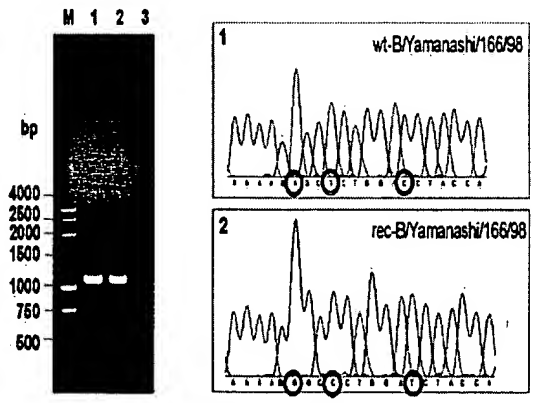

Fig. 3. Genetic characterization of recombinant influenza B viruses. RNA was isolated from allantoic fluid of chicken eggs either infected with wt B/Yamanashi/166/98 (1) or with supernatant of transfected COS7-MDCK cells with eight plasmids (2). RT-PCR was performed by using PA-specific primers. The PCR products were sequenced. The nucleotides in the electropherogram that differ between recombinant virus and wt virus are encircled.

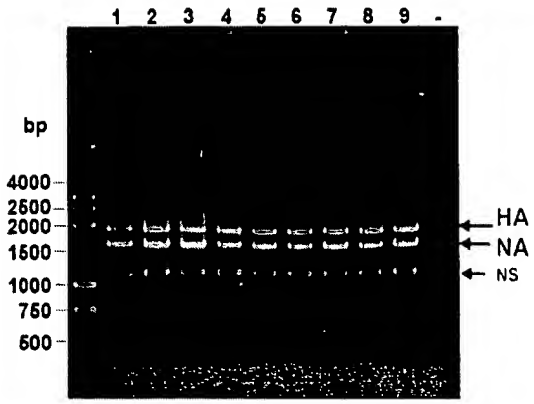

Fig. 4. RT-PCR for amplification of the HA and NA segments. RT-PCR was performed with the primer pair Bm-NAb-1/Bm-NAb-1557R. The PCR products were subjected to gel electrophoresis on a 1% agarose gel. RNA isolated from the following virus isolates were used: 1, B/Lee/40; 2, B/Ann Arbor/1/94; 3, B/Yamanashi/166/98; 4, B/Johannesburg/5/99; 5, B/Victoria/504/2000; 6, B/Sichuan/317/2001; 7, B/Shizuoka/15/2001; 8, B/Hawaii/10/2001; 9, B/Hong Kong/330/2001; –, no RNA.

Discussion

The fact that we are now able to rescue influenza A and influenza B virus reproducibly from a small number of plasmids containing the bidirectional RNA pol I-pol II transcription system proves the utility of this approach for generation of infectious virus entirely from cloned cDNA. Recently, the rescue of Thogoto virus, a tick-transmitted type D orthomyxovirus with a genome consisting of six negative-stranded RNA segments, was reported (34). This system required two plasmid sets: one set for the expression of the six vRNAs, which utilizes the human pol I promoter, and the other set for the expression of mRNAs for the viral structural proteins under the control of a T7 promoter. Expression of this latter set of RNAs required coinfection with a vaccinia virus expressing T7 RNA polymerase. Transfection of these 12 plasmids into 293T cells resulted in the efficient recovery of recombinant Thogoto virus.

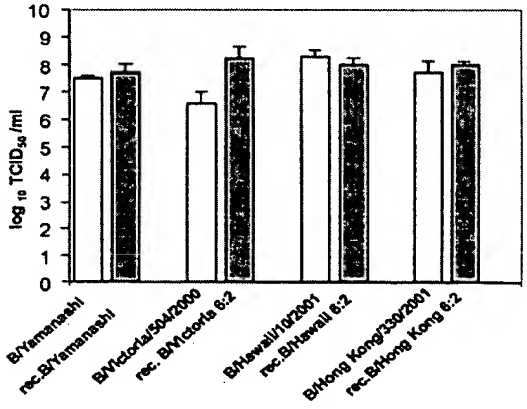

Fig. 5. Growth of 6 + 2 reassortants in eggs. Embryonated chicken eggs were inoculated with wt or recombinant virus. The virus titer was determined by titration on MDCK cells.

Possibly, the dual pol I-pol II promoter system could simplify and improve the Thogoto virus rescue by reducing the number of plasmids to six and eliminate the need for coinfection with vaccinia virus. The high efficiency of the dual promoter system for both type A and type B virus suggests that infectious influenza C virus, which consists of seven segments, can be rescued by cotransfecting seven pol I-pol II plasmids.

In principle, plasmid-only systems allow the manipulation of the noncoding and coding regions of the viral RNA. The non coding regions contain *cis*-acting signals for the regulation of transcription and replication of viral RNA (35, 36). Type-specific differences of the NCRs between members of the Orthomyxovirus family were analyzed in the last decade mostly by using *in vitro* transcription assays or *in vivo* reconstitution of vRNA-like templates encoding reporter genes such as chloramphenicol acetyltransferase (37–40). Those studies can now be extended to characterize sequence elements that are type specific and those that modulate gene expression and can be exchanged between types in the context of infectious viruses. Compared with influenza A virus, the NCRs of influenza B viruses are relatively large, extending up to 100 nt. By using the ribonucleoprotein transfection method (41) combined with an efficient antibody-driven selection method, the HA-NCR of influenza B virus was shown to be flexible in sequence and in length (42). Because the eight-plasmid system for influenza B virus does not require any selection procedure, similar mutagenesis studies can now be applied to all eight gene segments.

The classical method for generating influenza B inactivated vaccine virus is to grow the wt strain in embryonated chicken eggs. However, not all influenza B strains grow to high titers in eggs; therefore, occasionally a wt virus that is antigenically similar to, but not identical to, the circulating wt strain and that grows to acceptable titers in eggs is selected for vaccine production. The process of evaluating suitable vaccine candidates that meet these criteria is time consuming and laborious and may result in a candidate that lacks the optimal antigenicity. Here, we have demonstrated that the eight-plasmid system allows the generation of 6 + 2 reassortants expressing antigens from both influenza B lineages with the B/Yamanashi/166/98 backbone. These reassortants grew well in eggs and in most cases to higher titers than the wt parent expressing the HA and NA antigens.

The plasmid rescue system for influenza B virus described here should advance the reliability and quality of vaccines expressing appropriate HA and NA antigens. The consensus sequence of the recombinant transfectant virus was identical to the viral inserts in the plasmid clones. This high level of sequence integrity has been demonstrated with several other rescued type A viruses (43, 44). In addition, plasmid collections representing all antigenic variants of the B/Yamagata/16/88 and B/Victoria/2/87 lineages can now be created by using primers suitable for simultaneous RT-PCR of the HA and NA genes. Two to three weeks are required for sequencing and cloning of the HA and NA genes; subsequently, the reassortant viruses can be generated within 1 week. In addition to the faster generation of reassortants, using a standard master strain such as B/Yamanashi/166/98 may result in a more homogenous virus population with regard to the size and shape of virus particles. The growth of the reassortant viruses to high HA titers in eggs indicates that the growth is not restricted by incompatibilities between the six internal genes of B/Yamanashi/166/98 and the glycoproteins from the selected vaccine strains. Genetic engineering of the B/Yamanashi/166/98 plasmids or use of a different high growing master strain such as B/Lee/40, which has recently been rescued in our laboratory, may even increase the virus yield.

The plasmid rescue system for influenza B will enable molecular genetic studies of influenza B viruses. Influenza A and B viruses have distinctive molecular biological properties and produce different gene products. The advent of a molecular genetics system that allows recombinant viruses to be made without requiring a selective pressure should allow dissection of specific gene functions of influenza B virus. This system can be used to evaluate attenuating lesions in attenuated influenza B strains, such as B/Ann Arbor/1/66, and discover the requirement for specific gene products during the viral replication cycle.

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A Reverse Genetics Approach for Recovery of Recombinant Influenza B Viruses Entirely from cDNA

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The recovery of recombinant influenza A virus entirely from cDNA was recently described (9, 19). We adapted the technique for engineering influenza B virus and generated a mutant bearing an amino acid change E116G in the viral neuraminidase which was resistant in vitro to the neuraminidase inhibitor zanamivir. The method also facilitates rapid isolation of single-gene reassortants suitable as vaccine seeds and will aid further investigations of unique features of influenza B virus.

Influenza illness, a cause of more than 20,000 excess deaths in epidemic years in the United Kingdom (7), is attributable to infection with one of two subtypes of influenza A virus or with influenza type B virus (8). Thus, influenza B virus surface antigens are an essential component of any vaccine effective in reducing influenza morbidity. Influenza B viruses, like their type A counterparts, are orthomyxoviruses with genomes comprised of eight segments of negative-sense single-stranded RNA. In general, they encode proteins with homology to those encoded by influenza A virus, although there are notable and interesting differences, particularly in RNA segments 6 and 7 (16). In addition, a recently described additional open reading frame within RNA segment 2 present in many influenza A virus strains is absent from influenza B viruses (5). The reasons for and biological consequences of the genetic differences between influenza A and B viruses are of considerable academic interest and may have implications for understanding the limited host range for influenza B virus as well as for developing antiviral strategies for these orthomyxoviruses.

The manipulation of the influenza virus genome was first described by Palese and coworkers in 1989 (17), and the development of the technique to site-specifically change an infectious influenza B virus followed shortly after (1). However, this method, which utilized a helper virus and appropriate selection system, suffered from the limitation that engineering of all RNA segments was not possible. Since 1999 it has been possible to recover recombinant influenza A viruses entirely from cDNAs (9, 13, 19). Even within the short time this system has been available, it has revolutionized the potential for influenza virus research by facilitating a reverse genetic approach to the study of all the influenza A virus genes (3, 5, 11, 12, 20, 27). The process involves the in situ generation of virus RNAs transcribed from eight separate plasmids in which the influenza virus sequences are placed downstream of polymerase I promoters. In addition, expression of the four proteins which comprise the viral polymerase, NP, PB1, PB2, and PA, is achieved by placing their coding sequences under the control of a polymerase II promoter either in separate expression plasmids or in bidirectional plasmids (13). In this report we describe the adaptation of reverse genetics technology for engineering recombinant influenza B viruses.

The strategy we applied is based upon that described for recovery of recombinant influenza A viruses (9, 19). We used a cassette vector, pPRG, which allows cloning of the segments of the influenza B virus genome so that they are flanked by a human polymerase I promoter at the 5′ terminus and the hepatitis delta virus (HDV) antigenomic ribozyme at the 3′ terminus, such that their transcription results in negative-sense RNAs with exact viral-like termini. The polymerase I promoter was cloned from a human genomic library by PCR using primers based on published sequences (15). The ribozyme sequence was the antigenomic sense ribozyme of HDV (kindly provided by A. Ball). The plasmid pPRG was previously used for the rescue of influenza virus A/Victoria/3/75 H3N2 from cloned DNA (J. Daly, A. Cadman, W. Snowden, M. Tisdale, and T. Zurcher, Abstr. Options Control Influenza IV, abstr. W23-5, 2000). We inserted sequences encoding the influenza B virus-like model RNA HABCAT (1) and cotransfected this plasmid, pPRGCAT, with four plasmids directing expression of the influenza B virus polymerase complex. These helper plasmids were based on vector pCI (Invitrogen) and had coding sequences for NP, PB1, PB2, and PA derived from B/Panama/45/90 virus (14) inserted downstream of a cytomegalovirus (CMV) promoter. As a control we inserted HABCAT sequences into the pPolIRTSapI vector, described and kindly provided by Fodor et al. (9), to create pPRFCAT. We sequenced both polymerase I promoters and found no nucleotide differences between them, although they differed from published sequences (15). However, it should be noted that pPRFCAT contains the HDV genomic sense ribozyme. The data in Fig. 1 indicate that chloramphenicol acetyltransferase (CAT) expression following transfection of each construct varied. It may be that the efficiency of cleavage by either ribozyme affects expression. However, we cannot exclude the possibility that different sequences surrounding the promoter and ribozyme in the two vectors affect their efficiencies. Since the pPRG vector resulted in the most CAT protein produced, we chose this for insertion of the influenza B virus gene segments.

We also tested whether polymerase proteins from a different

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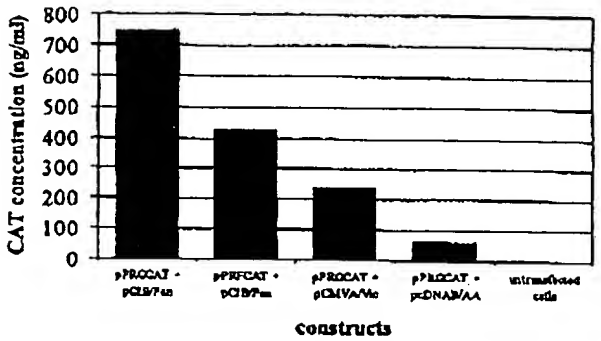


FIG. 1. Replication and expression of HABCAT influenza B virus model RNAs. CAT protein synthesized from two constructs containing an influenza B vRNA-like CAT reporter gene was quantified. pPRGCAT and pPRFCAT contain the CAT gene in a negative-sense orientation flanked by the influenza B virus HA gene noncoding regions between a human RNA polymerase I promoter and an HDV ribozyme terminator. One microgram of each construct was cotransfected into 5 × 10^5 293T cells with 0.5 μg of pCIPB1, 0.5 μg of pCIPB2, 0.5 μg of pCIPA, and 1 μg of pCINP. The pCI plasmids express B/Panama/45/90 polymerase and NP proteins. pPRGCAT was also cotransfected with plasmids expressing the A/Victoria/3/75 PB1, PB2, PA, and NP proteins or with pcDNA3-based plasmids expressing the same proteins from the B/Ann Arbor/66 virus. Transfections were performed in duplicate using the FuGENE 6 transfection reagent (Roche). After 48 h cells were lysed and cell lysates were used in a CAT enzyme-linked immunosorbent assay (Roche).

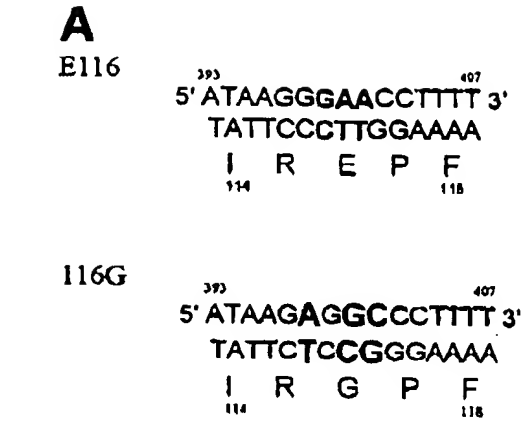
strain of influenza B virus, B/AA/66 (25), or from influenza A virus could support replication of the HABCAT viral-like RNA. Figure 1 shows that both supported replication of the model RNA, although less well than the B/Panama polymerase proteins. It is well established that influenza A virus polymerases replicate model RNAs containing influenza B virus promoters (6, 14, 18, 28). The differences we observed in replication efficiency by influenza B virus polymerases might be due either to sequence, since they are from different virus strains, or to the nature of the expression plasmid in which they were cloned. In virus rescue experiments we used B/Panama/45/90 polymerases (Table 1).

The influenza B virus gene segments were cloned by reverse transcription-PCR (RT-PCR), using appropriate primers complementary to the segment termini (26) (primer sequences available on request) that introduced *Bsm*BI or *Sap*I restriction enzyme sites to facilitate their insertion into pPRG cassette vectors containing these sites. Six of the RNA segments were derived from influenza virus B/Beijing/1/87. Segment 1 was derived from a cDNA clone of the PB2 gene from influenza virus B/Panama/45/90 (14). Segment 5 was obtained by RT-PCR from influenza virus B/Lee/40 viral RNA (vRNA). This cDNA was inserted into a bidirectional construct containing both polymerase I and polymerase II promoters by subcloning the polymerase I and ribozyme sequences into pcDNA3 (Invitrogen) (Table 1). Aliquots of 0.5 μg of each of the eight plasmids were cotransfected into 293T cells with B/Panama polymerase expression plasmids. Sixteen hours after transfection the 293T cells were cocultured with MDCK cells in the presence of 2.5 μg of trypsin/ml. Cytopathic effect (CPE) was observed 71 h postcoculturing, and subsequent rescue experiments confirmed that CPE routinely appears between 68 and 72 h postcoculture. Cell supernatant harvested after a further 36 h displayed a titer of 32 hemagglutinating units and an infectivity of approximately 10^6 PFU/ml. Analysis of RNA segments of the recovered virus by RT-PCR, diagnostic restriction enzyme digestion, and sequencing of the PCR products illustrated that the recovered virus contained a gene constellation dictated by the plasmids used for transfection and hitherto unreported in our laboratories or in the literature. The rescued virus contains a B/Lee/40 virus nucleoprotein (NP) gene which is easily identified since it differs from other influenza B virus NP genes in that it contains a single *Eco*RI restriction site, whereas other sequences contain two or more, and it lacks a *Bsm*BI restriction site whereas this site is present at nucleotide 1370 in other influenza B viruses. Moreover, influenza virus B/Panama/45/90 virus has never been propagated in our laboratory, and the sequence of RT-PCR products from the recombinant virus illustrates that it contains a B/Panama/45/90 PB2 segment (data not shown).

We believe this to be the first successful recovery of recombinant influenza B virus entirely from cDNA. Although the technique we have used is an adaptation of that previously developed for influenza A viruses, we have made some important and possibly significant improvements. Firstly, as discussed above, the plasmid vector and polymerases used in the system differ from those used in other laboratories. Secondly, the genetic constellation of the virus we rescued is unusual (Table 1). Six of the segments are from influenza virus B/Beijing/1/87 virus, but PB2 is from B/Panama/45/90 and NP is from the highly laboratory-adapted strain B/Lee/40. This was useful for identifying the recovered virus and also may contribute to our success, as it results in a virus which replicates very well in MDCK cells, so even if transfection efficiency were low recovered virus would be readily amplified following coculture. Thirdly, we utilized a bidirectional vector for generation of segment 5 RNA. We do not know at present whether this is essential for virus recovery. However, subcloning of segment 5 cDNAs into the pPRG vector did not allow recovery of virus. Interestingly, although the bidirectional plasmid generates NP that can support HABCAT model RNA replication (data not shown), omission of the pCINP plasmid did not allow virus recovery, although substitution with pcDNA3NP from B/AA/66 did.

TABLE 1. Plasmids used for rescue of recombinant influenza B virus entirely from cDNA

Plasmid	Origin of viral genes	Promoter
pPRPB1	B/Beijing/1/87	Human RNA polymerase I
pPRPB2	B/Panama/45/90	Human RNA polymerase I
pPRPA	B/Beijing/1/87	Human RNA polymerase I
pPRHA	B/Beijing/1/87	Human RNA polymerase I
pPRNP	B/Lee/40	Human RNA polymerase I and CMV RNA polymerase II
pPRNA	B/Beijing/1/87	Human RNA polymerase I
pPRM	B/Beijing/1/87	Human RNA polymerase I
pPRNS	B/Beijing/1/87	Human RNA polymerase I
pCIPB1	B/Panama/45/90	CMV RNA polymerase II
pCIPB2	B/Panama/45/90	CMV RNA polymerase II
pCIPA	B/Panama/45/90	CMV RNA polymerase II
pCINP	B/Panama/45/90	CMV RNA polymerase II



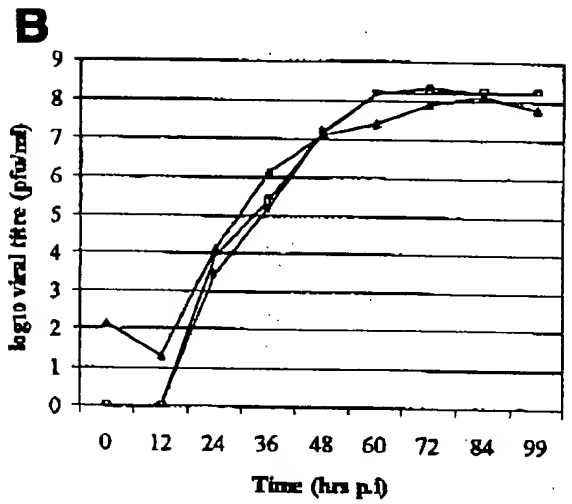


FIG. 2. (A) Nucleotide and amino acid sequences of B⁺WNP, B⁺E116G and B⁺HA2X rescued virus NA. B⁺WNP and B⁺HA2X contain the E116 sequence and B⁺E116G contains the 116G sequence. The bold lettering in the E116 nucleotide sequence highlights the codon coding for glutamic acid at amino acid position 116. The bold lettering in the 116G sequence highlights single base mutations (introduced into the pPRNA plasmid via site-directed mutagenesis) within the NA gene of B⁺E116G rescued virus. (B) Multiple-step growth curve of B⁺WNP (●), B⁺E116G (□), and B⁺HA2X (▲) rescued viruses in MDCK cells. Eight 3.5-cm² wells of confluent MDCK cells (approximately 10^6 cells) were infected with each virus at a multiplicity of infection of 0.001 for 1 h at 34°C. Cells were washed, and serum-free Dulbecco's modified Eagle's medium containing 2.5 μg of trypsin/ml was added to each well, followed by incubation at 34°C. Cell supernatant from one well was harvested after each of the following time points: 12, 24, 36, 48, 60, 72, 84, and 99 h postinfection (p.i).

We engineered a mutation into the pPRNA plasmid to alter amino acid residue 116 of the B/Beijing/1/87 neuraminidase (NA) protein from glutamic acid to glycine (Fig. 2A). This mutation has been independently identified in two laboratory isolates which acquired resistance to the NA inhibitor zanamivir (2, 24). A virus was recovered that grew well in MDCK cells. Figure 2B illustrates the multicycle growth characteristics in comparison with the recovered wild-type virus with the same genetic constellation. As predicted, the E116G mutant displayed a drug-resistant phenotype in plaque reduction assays. At a concentration of 0.03 μg of zanamivir/ml, the size and number of plaques formed by wild-type virus were greatly reduced but the E116G mutant was unaffected. Sequencing of RT-PCR products confirmed that the E116G mutation was present, as was a silent mutation in the preceding codon engineered into the cDNA as a tag (Fig. 2A).

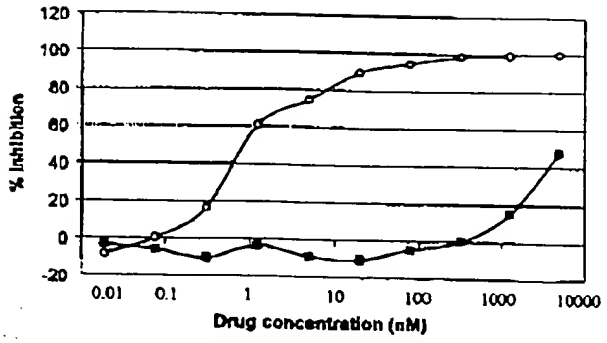


FIG. 3. Inhibition of B⁺WNP (○) and B⁺E116G (■) rescued virus NA activity by zanamivir via the adaptation of the NA assay of Potier et al. (21).

We next assayed the inhibition of mutant NA enzyme activity by zanamivir. Infected cell supernatants containing wild-type or E116G virus were adjusted for equivalent NA activity and then subjected to the 4-methylumbelliferyl-α-D-acetylneuraminate (MUNANA) enzyme assay in the presence of different concentrations of drug. A greater amount of virus was needed to assay the mutant enzyme, indicating that it had a reduced NA activity. Indeed, hemagglutination elution at 37°C (assumed to be mediated by NA activity) was not observed for the mutant virus. Moreover, the E116G mutant NA was only inhibited by high concentrations of zanamivir. The 50% inhibitory concentration (IC_{50}) was approximately 1 nM for wild-type virus but increased to over 8 μM for the mutant (Fig. 3). These results correlate well with IC_{50} values previously obtained for wild-type and E116G B/Beijing/1/87 viruses (2).

Since both influenza B viruses previously described which contain the E116G mutation also contained additional hemagglutinin (HA) mutations (2, 24), we sequenced RT-PCR products derived from the entire HA gene of the mutant virus. No changes from the wild-type B/Beijing/1/87 HA sequence were found. Thus, the reverse genetics procedure has allowed the recovery of a genetically defined virus altered in a single gene segment which can help to understand the contribution of single point mutations to a drug resistance phenotype.

It has been particularly difficult to study the contribution of NA mutations to resistance to the NA inhibitors in the context of live virus when using a traditional genetic approach, since NA mutations are always accompanied by changes in the HA gene both in vitro and in vivo (2, 10, 24). In studies with influenza A virus mutants, the contributions of the NA and HA sequence changes to the drug-resistant phenotype have been separated by generating single-gene reassortants (4). The reverse genetics technology allows the analysis of individual mutations in separate gene segments without needing to resort to such methods. The NA E116G mutant demonstrates that the

drug-resistant phenotype to an NA inhibitor can be conveyed by a single amino acid change in the NA gene. Interestingly, the E116G virus displayed efficient replication in MDCK cells. However, it has been shown previously that NA mutants with reduced enzyme activity may grow well in MDCK cells but have reduced infectivity in vivo (11; J. Carr, J. Ives, N. A. Roberts, C. Y. Tai, D. B. Mendel, L. Kelly, R. Lambkin, and J. Oxford, Abstr. 2nd Int. Symp. Influenza Other Respir. Viruses, 1999). It may be that the reduction in NA activity is not sufficient to significantly disrupt the balance between HA affinity and NA activity in MDCK cells. Changes in HA leading to altered receptor binding and antigenic properties of influenza B viruses upon adaptation to cell culture and eggs have been documented (22, 23).

We replaced the pPRHA plasmid with pPRHA2X, which encodes the HA gene from influenza virus B/Md/59 genetically tagged with an *Xba*I restriction enzyme site (1). A recombinant virus was rescued which contained the genetic tag and displayed similar growth kinetics to the wild type and the drug-resistant mutants described above (Fig. 2B).

The ability to generate antigenic variants of influenza B virus within a high-growth background of internal genes may have important consequences for generation of vaccine strains. In recent years the World Health Organization (WHO)-recommended strain of influenza B virus has grown poorly in eggs. During 1998 to 2002, alternative influenza B virus strains which resembled the WHO strain antigenically but grew to higher titers in eggs were selected for use in EU vaccines (John Wood, personal communication). Even so, in some cases growth of the chosen virus was still inefficient, causing a potential reduction in the number of available doses. Since typically influenza A virus high-growth reassortants increase yield approximately fourfold over that of wild-type strains, the ability to generate similar high-growth strains of influenza B virus to order will be a useful addition to the influenza virus reverse genetic repertoire.

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ADDENDUM IN PROOF

Since submission of this article, Hoffmann et al. have reported the rescue of recombinant influenza B virus using a similar approach (E. Hoffmann, K. Mahmood, C.-F. Yang, R. G. Webster, H. B. Greenberg, and G. Kemble, Proc. Natl. Acad. Sci. USA 99:11411–11414, 2002).

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Rescue of Recombinant *Thogoto Virus* from Cloned cDNA

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***Thogoto virus* (THOV) is a tick-transmitted orthomyxovirus with a genome consisting of six negative-stranded RNA segments. To rescue a recombinant THOV, the viral structural proteins were produced from expression plasmids by means of a vaccinia virus expressing the T7 RNA polymerase. Genomic virus RNAs (vRNAs) were generated from plasmids under the control of the RNA polymerase I promoter. Using this system, we could efficiently recover recombinant THOV following transfection of 12 plasmids into 293T cells. To verify the recombinant nature of the rescued virus, specific genetic tags were introduced into two vRNA segments. The availability of this efficient reverse genetics system will allow us to address hitherto-unanswered questions regarding the biology of THOV by manipulating viral genes in the context of infectious virus.**

Thogoto virus (THOV) is the prototype tick-transmitted orthomyxovirus (18). The genome of THOV consists of six single-stranded RNA segments of negative polarity that are encapsidated by the viral nucleoprotein (NP) and associate with the viral RNA polymerase complex to form ribonucleoprotein complexes (vRNPs) (4, 17). Each individual segment codes for a single structural protein: the three subunits of the viral RNA polymerase complex (PB2, PB1, and PA) (11, 25), the viral surface glycoprotein (GP) (12), the NP (26), and the matrix protein (M) (10). Members of the genus *Thogotovirus* are structurally and genetically similar to the influenza viruses but are unique in their ability to infect mammalian as well as tick cells (15). The host change between vertebrates and arthropodes requires specific adaptations to allow the virus to replicate in both cell types. Accordingly, THOV has unique features like the single GP that has no similarities to the influenza virus glycoproteins but has similarity with the surface glycoproteins of baculoviruses (12). In addition, THOV has a unique cap-snatching mechanism, using only the cap structure and one additional nucleotide from cellular mRNAs to initiate viral transcription (2, 26). Moreover, the genome of THOV does not encode additional proteins, like the NS2/NEP or the NS1 of influenza A virus (FLUAV). NS2/NEP is essential for the export of the newly synthesized vRNPs out of the nucleus (13, 16). The nonstructural protein NS1 has been shown to suppress interferon production and the interferon-mediated antiviral response of the infected host cell, most likely by sequestration of double-stranded RNA molecules (7, 23). Since THOV lacks analogous proteins, it depends on the basic set of its six structural proteins to perform nuclear export of the vRNPs and to deal with the interferon-dependent suppression of viral replication. Specific manipulations of the THOV genome should allow to assign such functions to defined viral genes.

We recently succeeded in generating THOV-like particles (24). In this system, synthesis of the six structural THOV proteins together with a model minigenome RNA was sufficient for the formation of functional vRNPs and assembly of infectious virus-like particles. Here, we modified this system by expressing all six genomic vRNA segments from RNA polymerase I-driven expression plasmids instead of the model minigenome. This modification allowed us to rescue infectious recombinant THOV (recTHOV) entirely from cloned cDNAs.

MATERIALS AND METHODS

Plasmid constructs. The structural proteins of THOV were produced from the expression vectors pG7-PB2, pBS-PB1, pBS-PA, pBS-GP, pG7-NP, and pBS-M, all under the control of the T7 RNA polymerase promoter, as described previously (24, 27). These cDNA plasmids were used as templates to generate RNA polymerase I constructs for the expression of the full-length genomic segments of THOV. The cDNAs were amplified by PCR using primers containing *Bsm*BI restriction sites and sequences corresponding to the 3′ and 5′ noncoding sequences of the genomic segments (all accession numbers are from GenBank): segment 1, nucleotides (nt) 1 to 14 and 2325 to 2375 (accession no. Y17873); segment 2, nt 1 to 25 and 2159 to 2212 (accession no. AF004985); segment 3, nt 1 to 20 and 1890 to 1927 (accession no. AF006073); segment 4, nt 1 to 15 and 1555 to 1574 (accession no. M77280); segment 5, nt 1 to 20 and 1386 to 1418 (accession no. X96872); and segment 6, nt 1 to 20 and 937 to 956 (accession no. AF236794). The sequences of the primers will be provided on request. The PCR products were digested with *Bsm*BI and inserted into the *Bsm*BI site of pHH21 between the human RNA polymerase I promoter and terminator regions (kindly provided by Gerd Hobom, Justus Liebig University, Giessen, Germany) (14), yielding pHH21-vPB2, pHH21-vPB1, pHH21-vPA, pHH21-vGP, pHH21-vNP, and pHH21-vM. To introduce silent mutations into the cDNA of pHH21-vGP and pHH21-vNP, we amplified two overlapping cDNA fragments with a common *Kpn*I or *Nsi*I site, respectively. For the PCR, we combined primer S4/PstI (H45; nt 1273 to 1310) (5′ CTCTGGTACCCTTCTGCAGCCGAAGTCGATTTTAG GGG 3′) and the reverse-sense counterpart (H46; nt 1263 to 1286) with the primers coding for the noncoding regions of segment 4 and primer S5/ClaI (H41; nt 631 to 660) (5′ GGAAATCGATCGTCGGGCACCTCAAGCGCC 3′) and the reverse-sense counterpart (H42; nt 611 to 645) with the primers coding for the noncoding regions of segment 5. These internal primers introduced a unique *Pst*I restriction site into the segment 4 cDNA at position 1290 and a second *Cla*I restriction site into segment 5 cDNA at position 637. The PCR products were digested with *Bsm*BI/*Kpn*I for segment 4 and *Bsm*BI/*Nsi*I for segment 5 and inserted into pHH21 in a three-molecule ligation reaction. The sequences of all PCR-derived cDNA constructs were confirmed by sequencing.

Cells, viruses, and antibodies. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. We used 293T human embryonic kidney cells for transfection and African

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green monkey kidney (Vero) cells and BHK-21 cells for the cultivation of the viruses.

Thogoto virus strain SiAr126 (wild-type THOV) (1) was used as a control. The recombinant MVA-T7 vaccinia virus expressing the T7 RNA polymerase was kindly provided by Gerd Sutter (GSF, Neuherberg, Germany) (22).

A polyclonal hyperimmunized guinea pig antiserum directed against THOV proteins (kindly provided by P. A. Nuttall, NERC Institute of Virology and Environmental Microbiology, Oxford, United Kingdom) (9) was used for the neutralization experiments.

Generation of recTHOV. A monolayer of 293T cells (10^6 cells in 35-mm-diameter dishes) was infected with 10 PFU of MVA-T7 per cell for 1 h at 37°C. Then, the cells were transfected with the T7 expression plasmids and the RNA polymerase I expression plasmids with LipofectAMINE 2000 (Gibco BRL). The 12 plasmids were used in the following quantities: 500 ng of pG7-PB2, 500 ng of pBS-PB1, 500 ng of pBS-PA, 500 ng of pBS-GP, 2.5 μg of pG7-NP, 250 ng of pBS-M, and 500 ng of each pHH21 expression plasmid. After 5 h, the transfection solution was replaced with 1 ml of DMEM with 5% fetal calf serum and 20 mM HEPES (pH 7.3), and the cells were further incubated at 37°C for 4 to 5 days. The supernatant was then collected, cleared from cell debris, and passaged onto a monolayer of Vero cells (3×10^6 in 60-mm-diameter dishes). After 1 h of virus attachment, the inoculum was exchanged for 5 ml of DMEM with 5% fetal calf serum and 20 mM HEPES (pH 7.3), and the cells were further incubated for 5 days or until a cytopathic effect was visible. recTHOV present in the supernatant was subjected to plaque purification on Vero cells.

Genetic analysis of recTHOV. To detect the silent mutations introduced into segments 4 and 5, the supernatants of Vero cells (10^6 cells in a 35-mm-diameter dish) infected with wild-type THOV or recTHOV were used to isolate vRNAs from polyethylene glycol-precipitated virus particles. A total of 1.25 ml of supernatant was mixed with 250 μl of polyethylene glycol 8000 (40% in 2.5 M NaCl). The mixture was incubated for 30 min on ice and then spun down in a microcentrifuge at 15,000 × *g* for 20 min. The pellets were resuspended in 200 μl of a solution containing 10 mM Tris (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, and 0.3% sodium dodecyl sulfate. A total of 200 μl phenol-chloroform-isoamyl alcohol (25:24:1) was added, and the samples were incubated at 56°C for 10 min with occasional mixing. The RNA was precipitated from the aqueous phase with ethanol. vRNAs were reverse transcribed using primer H66 (nt 481 to 500) for segment 4 and primer H71 (nt 467 and 486) for segment 5, and the cDNAs were then amplified by PCR with primers specific for segment 4 (nt 481 to 500 and 1536 to 1574) and segment 5 (nt 467 to 486 and 1373 to 1418). The reverse transcriptase (RT)-PCR products were analyzed for the presence of the novel restriction sites by digestion of the PCR products of segment 4 with *Pst*I and the PCR products of segment 5 with *Cla*I.

Growth properties and virus plaque assay. To determine the growth characteristics of recTHOV, the viruses of two independent transfection experiments were plaque purified and used to prepare virus stocks on Vero cells. In parallel, virus stocks of wild-type THOV were prepared from plaque-purified viruses. BHK cells in 25-cm^2 flasks were infected with these plaque-purified wild-type and recTHOV isolates at a multiplicity of infection of 0.01 PFU per cell and incubated at 37°C. At different time points, the supernatants were assayed for infectious virus by titration on Vero cells. The virus titers were calculated as reciprocals of the 50% tissue culture infective dose per ml. For plaque assays, monolayers of Vero cells in six-well macroplates (35 mm) were infected with about 100 PFU of THOV. For plaque reduction assays, the viruses were incubated on ice with a partially neutralizing solution of guinea pig antiserum for 60 min prior to infection. After incubation at 37°C for 1 h, the virus inoculum was removed, and medium containing 2% fetal calf serum, 20 mM HEPES (pH 7.3), 0.4% Noble agar, and 0.002% DEAE-dextran was added. After incubation at 37°C for 4 days, the agar overlay was removed, and the cells were stained with a solution of 1% crystal violet, 3.6% formaldehyde, 1% methanol, and 20% ethanol.

RESULTS AND DISCUSSION

Rescue of infectious recTHOV. To generate recTHOV, we cotransfected the full set of the six RNA polymerase I constructs encoding the six individual viral genomic RNA segments in negative-sense orientation together with the six T7 expression plasmids encoding the THOV structural proteins into 293T cells which were infected with MVA-T7 (Fig. 1). Infection with the attenuated recombinant vaccinia virus MVA-T7 (22) provided the bacteriophage T7 RNA polymerase necessary to synthesize the structural proteins of THOV. MVA-T7 infection did not cause cytopathic effects, and no progeny vaccinia viruses were produced. After 4 days, the supernatants of the transfected cells were passaged onto Vero cells known to be highly permissive for THOV (6). Plaque formation on Vero cells revealed that we were able to rescue recTHOV in most transfection experiments.

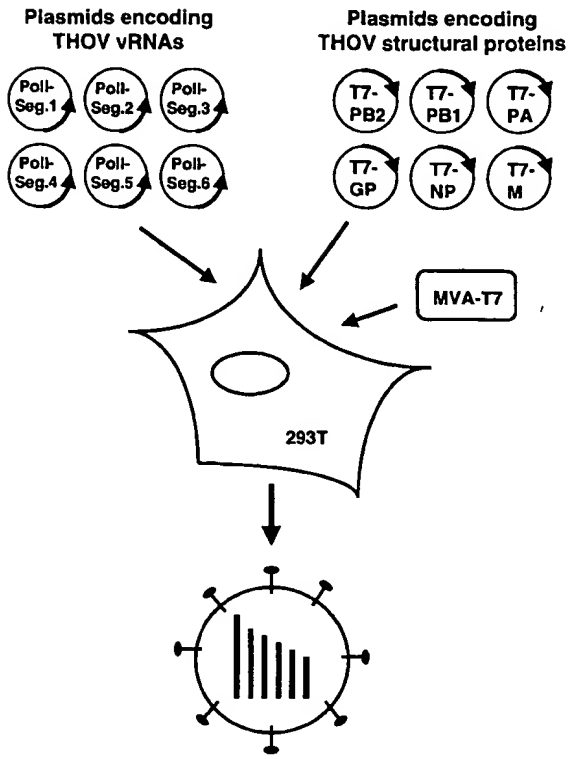

FIG. 1. Reverse genetics system for the generation of recTHOV. Twelve plasmids were transfected into MVA-T7-infected 293T cells. The six genomic negative-sense RNA segments were produced from expression plasmids containing the human RNA polymerase I promoter (Poll-Seg. 1 to 6). The six structural proteins of THOV were synthesized from expression plasmids under the control of the T7 promoter; the T7 RNA polymerase was provided by a recombinant vaccinia virus, MVA-T7. Infectious recTHOV was generated and released into the cell supernatant.

To monitor the time course of recTHOV production, aliquots of the supernatants of the transfected cells were removed every 24 h and titrated for infectivity. Table 1 summarizes the results of these experiments. Transfection of the full set of 12 plasmids into 10^6 cells yielded recTHOV between 48 and 96 h (Table 1, experiments 1 and 3). In most experiments, the initial titers of recombinant virus increased up to 10^7 PFU/ml, probably due to amplification of newly formed recombinant viruses in the 293T cell culture; whereas in some experiments, no infectious virus could be detected (Table 1, experiment 2).

In principle, synthesis of the three subunits of the vRNA polymerase and the nucleoprotein together with the genomic vRNAs should be sufficient to generate recombinant viruses, as has been demonstrated for FLUAV (5, 14). This would allow the omission of the T7 expression constructs coding for GP and M from the set of transfected plasmids. We attempted to rescue recTHOV by omitting the T7 expression plasmid coding

TABLE 1. Generation of recTHOV, following plasmid transfection of 293T cells

Hours after plasmid transfection	Virus titers in culture supernatant (PFU/ml) in expt[a]:				
	1 (complete)	2 (complete)	3 (complete)	4 (−T7/M)	5 (−T7/M)
24	0	0	0	0	0
48	10	0	0	0	0
72	1.1×10^4	0	0	0	0
96	1×10^6	0	90	0	1
120	1.5×10^7	0	2.4×10^3	1	2.4×10^3
144	7.5×10^7	0	4.4×10^4	3×10^3	8.7×10^4
172	5×10^7	0	1.5×10^6	4.6×10^4	2×10^7

[a] 293T cells were transfected with the full set of 12 expression plasmids (complete) or with all plasmids except the M-encoding helper plasmid (−T7/M). At different time points, the virus titer of the culture supernatants was determined.

for M. This also led to the formation of progeny (Table 1, experiments 4 and 5). Similarly, we were able to recover recTHOV by using only the four expression plasmids coding for the three polymerase subunits and NP (data not shown).

In the rescue system described here, two established protocols for the rescue of negative-strand RNA viruses were combined. The T7 RNA polymerase was used to produce the required viral proteins in high quantities. The cellular RNA polymerase I expression system was used to provide vRNA molecules with the correct 3′ and 5′ ends of the authentic viral genome segments, as described for the rescue of FLUAV (5, 14). It should be noted that recTHOV was rescued from RNA polymerase I expression plasmids producing negative-sense vRNAs. This is in contrast to earlier studies describing the recovery of recombinant negative-strand RNA viruses using positive-sense, antigenomic RNAs (3, 19, 20, 21). In these studies, expression of positive-sense antigenomic RNAs was chosen to avoid any risks of hybridization with the mRNA transcripts coding for the support proteins. In our rescue system, formation of such double-stranded RNA species was presumably prevented by physically separating the synthesis of mRNA transcripts from that of vRNA transcripts within the cytoplasmic and nuclear compartments. Recently published systems to generate recombinant FLUAV used nuclear RNA polymerase I to express the vRNA segments and nuclear RNA polymerase II to produce mRNAs (5, 8, 14), suggesting that, at least for FLUAV, the simultaneous expression of positive-sense mRNA and negative-sense vRNA species in the same cellular compartment was not a problem for the rescue of recombinant viruses.

Identification of the genetically tagged recTHOV. To prove that the rescued virus was derived from the transfected cDNAs and did not represent a laboratory contamination, we introduced silent mutations into the cDNAs encoding segments 4 and 5. The altered nucleotide sequences resulted in a new *Pst*I restriction site in segment 4 and a second *Cla*I restriction site in segment 5. In both cases, the amino acid sequence of the encoded viral proteins was not altered. To identify the silent mutations, recTHOV obtained after transfection of the 12 plasmids was plaque purified and propagated on Vero cells.

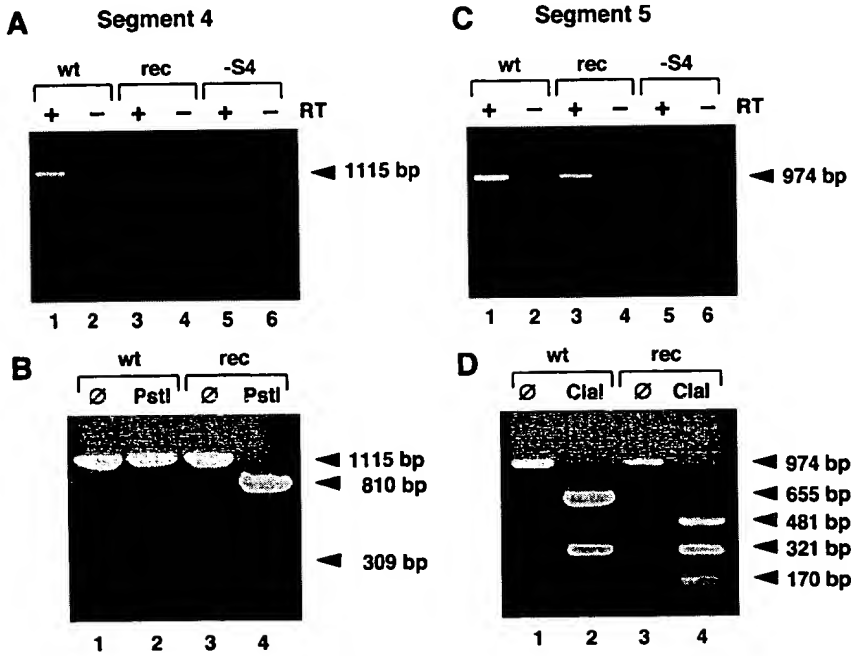

FIG. 2. recTHOV carries genetic markers. vRNA was isolated from virus particles obtained from supernatants of recTHOV-infected (rec) or wild-type THOV-infected (wt) cells. As a control, supernatants of Vero cells treated with the supernatants of 293T cells which had been transfected with plasmids for all vRNA segments except segment 4 (-S4) were used for RNA isolation. Segment 4 (A and B) and segment 5 (C and D) genomic vRNAs were detected by RT-PCR with primers that allow the amplification of a 1,115-bp segment 4 fragment (position 481 to 1574) and a 974-bp segment 5 fragment (position 467 to 1418). RT-PCRs without RT enzyme (−RT) were used as controls. The presence of the newly created *Pst*I site in the cDNA of segment 4 (B) and of the additional *Cla*I restriction site in the cDNA of segment 5 (D) of the recTHOV was determined by restriction analysis of the RT-PCR products. The products were analyzed by agarose gel electrophoresis in the presence of ethidium bromide. The molecular sizes of the fragments are indicated at the right.

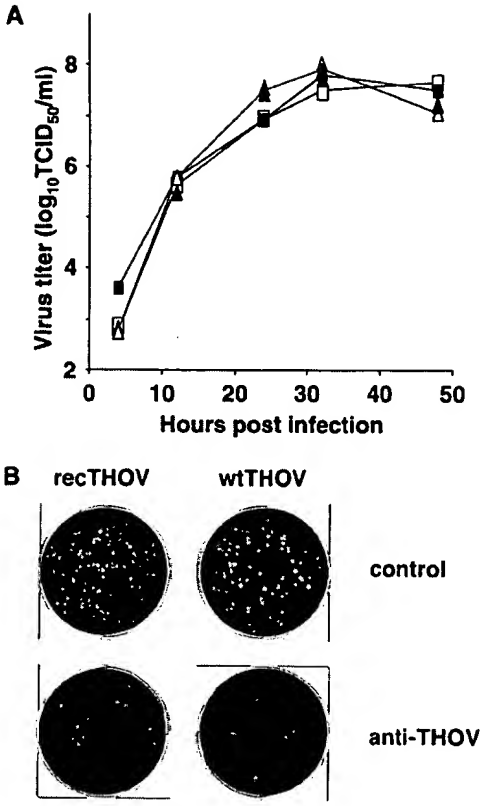

FIG. 3. Growth characteristics of recTHOV. (A) Growth curves of recTHOV and wild-type THOV. BHK cells were infected with plaque-purified isolates of THOV at a multiplicity of infection of 0.01 PFU per cell. At the indicated times after infection, the virus titer in the supernatant was determined. □, wild-type isolate 1; ■, wild-type isolate 2; △, recTHOV isolate 1; ▲, recTHOV isolate 2. (B) Plaque formation of recTHOV. Vero cell monolayers were infected with about 100 PFU of recTHOV or wild-type THOV and incubated under soft agar. For neutralization, 100 PFU of wild-type THOV or recTHOV was preincubated with a guinea pig antiserum directed against THOV for 60 min before infection of Vero cells. After 4 days, cells were fixed and stained with crystal violet.

The progeny virus was harvested from the cell supernatant, and genomic RNA was extracted. The vRNA preparation was used to amplify short cDNA fragments by RT-PCR using primers specific for segments 4 and 5. In parallel, the same protocol was applied to wild-type virus. Analysis of the RT-PCR products by agarose gel electrophoresis revealed cDNA fragments of the expected sizes of 1,115 and 974 bp for segments 4 and 5, respectively (Fig. 2A and C, lanes 1 and 3). Amplification of the same vRNA samples without the RT step failed to produce positive signals (Fig. 2A and C, lanes 2 and 4), excluding the presence of cDNA contaminations in the vRNA preparations. As a further control, cells were transfected with the full set of expression plasmids except that for vRNA segment 4. The supernatant of this transfection experiment was passaged onto Vero cells, and the resulting supernatant was treated exactly as described for the isolation of vRNA from virus-infected cells. Analysis of this RNA preparation did not show any signal in the RT-PCR (Fig. 2A and C, lanes 5 and 6), as expected. Next, the RT-PCR products were incubated with the appropriate restriction enzymes. Treatment of the PCR product derived from segment 4 of recTHOV with *Pst*I resulted in two fragments, whereas only one band, corresponding to the uncleaved PCR product, was detected in the case of wild-type THOV (Fig. 2B). Similarly, digestion of the RT-PCR products of segment 5 with *Cla*I revealed the presence of the extra *Cla*I restriction site in the cDNA derived from recTHOV but not that from wild-type THOV (Fig. 2D). These results demonstrate that the rescued virus was a true recombinant virus derived from the transfected cDNAs.

Characterization of recTHOV. We compared the growth properties of the rescued virus with that of wild-type THOV in BHK cells (Fig. 3A). Wild-type THOV and recTHOV from two independent transfection experiments were plaque purified. Stocks derived from these purified viruses were used to infect BHK cells. Yields of progeny viruses in the culture supernatants were determined at different time points postinfection. The recTHOV isolates and the wild-type viruses produced titers of about 4×10^7 to 8×10^7 50% tissue culture infective doses per ml of the culture supernatant at 32 h postinfection. Clearly, recTHOV did not differ appreciably from the wild-type virus in either growth rate or the maximal titer reached.

To further characterize the rescued virus, we performed plaque assays on Vero cell monolayers. As shown in Fig. 3B, the plaque size of wild-type and recTHOV was approximately equal, again indicating that the two viruses had comparable growth characteristics. We further compared the neutralizing capacity of a polyclonal antiserum directed against THOV by preincubating about 100 PFU of recTHOV or wild-type THOV with the antibody before testing for infectivity by plaque assay. The antiserum was used at a dilution to allow some breakthrough of the viruses. Growth of both viruses was reduced to a similar degree, indicating that recTHOV and wild-type THOV are antigenically identical (Fig. 3B).

In summary, we have established an efficient system for the rescue of recTHOV entirely from cDNA without the need of a homologous helper virus. Our system combines the strong T7-driven synthesis of the viral structural proteins with the RNA polymerase I-dependent expression of the six genomic RNA segments. The recovered recTHOV showed properties similar to that of authentic wild-type THOV. Therefore, THOV is the second orthomyxovirus for which a reverse genetics system is now available. This system will allow the study of THOV-specific aspects of the orthomyxovirus life cycle by observing the effects of specific mutations in the viral genome. It can be used to study open questions of the biology of THOV: the importance of the baculovirus-like GP of THOVs for the host change between mammals and ticks or the influenza C virus-like coding strategy of the THOV M (10). In addition, questions about virally encoded activities like the NS2/NEP-dependent nuclear export pathway or the M2 ion channel activity, which are essential for FLUAV multiplication but seem to be dispensable for THOV, can now be studied in the context of recTHOV.

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Reverse Genetics System for Uukuniemi Virus (*Bunyaviridae*): RNA Polymerase I-Catalyzed Expression of Chimeric Viral RNAs

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We describe here the development of a reverse genetics system for the phlebovirus Uukuniemi virus, a member of the *Bunyaviridae* family, by using RNA polymerase I (pol I)-mediated transcription. Complementary DNAs containing the coding sequence for either chloramphenicol acetyltransferase (CAT) or green fluorescent protein (GFP) (both in antisense orientation) were flanked by the 5′- and 3′-terminal untranslated regions of the Uukuniemi virus sense or complementary RNA derived from the medium-sized (M) RNA segment. This chimeric cDNA (pol I expression cassette) was cloned between the murine pol I promoter and terminator and the plasmid transfected into BHK-21 cells. When such cells were either superinfected with Uukuniemi virus or cotransfected with expression plasmids encoding the L (RNA polymerase), N (nucleoprotein), and NSs (nonstructural protein) viral proteins, strong CAT activity or GFP expression was observed. CAT activity was consistently stronger in cells expressing L plus N than following superinfection. No activity was seen without superinfection, nor was activity detected when either the L or N expression plasmid was omitted. Omitting NSs expression had no effect on CAT activity or GFP expression, indicating that this protein is not needed for viral RNA replication or transcription. CAT activity could be serially passaged to fresh cultures by transferring medium from CAT-expressing cells, indicating that recombinant virus containing the reporter construct had been produced. In summary, we demonstrate that the RNA pol I system, originally developed for influenza virus, which replicates in the nucleus, has strong potential for the development of an efficient reverse genetics system also for *Bunyaviridae* members, which replicate in the cytoplasm.

The procedures developed during the 1990s to genetically manipulate the genomes of negative-strand viruses and to rescue infectious viruses entirely from cloned cDNAs, commonly referred to as reverse genetics, have revolutionized the analyses of viral gene expression and the dissection of *cis*-acting regulatory sequences important for replication and transcription. They have also paved the road for engineering these viruses for vaccine and gene therapy purposes (6, 36). The ability to rescue infectious viruses from cloned cDNAs has by now been well established for nonsegmented, negative-strand viruses (*Mononegavirales*), such as members of the *Rhabdoviridae* (22, 41, 48) and *Paramyxoviridae* (1, 5, 18, 19, 35) families. The development of similar protocols for manipulating the genomes and creating viruses from cloned cDNAs of segmented, negative-strand viruses, i.e., members of the *Orthomyxoviridae*, *Bunyaviridae*, and *Arenaviridae* families, have turned out to be much more difficult. Although the ability to manipulate RNA segments of influenza A viruses was developed more than a decade ago (10, 25), it was not until last year that the first reports on the rescue of infectious influenza A virus entirely from cloned cDNAs were published (15, 20, 31, 32).

Members of the *Bunyaviridae* family, which comprises more than 300 viruses (28) grouped into the five genera *Bunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus*, are enveloped viruses with a tripartite, single-stranded RNA genome of negative polarity. The L segment encodes the RNA-dependent RNA polymerase (L), the M segment encodes the two spike proteins (G1 and G2) and in some viruses a nonstructural protein (NSm), while the S segment encodes the nucleoprotein (N) and in some viruses a nonstructural protein (NSs) (8, 40). Initiation of transcription of the viral mRNAs is primed by short sequences derived from the 5′ end of host mRNAs (2, 40, 43). This cap-snatching mechanism is reminiscent of that first described for influenza virus (21), with the important difference that cap snatching occurs in the cytoplasm of *Bunyaviridae*-infected cells, as opposed to the nucleus in influenza virus-infected cells. This is due to the fact that *Bunyaviridae* members replicate exclusively in the cytoplasm. As is the case for all negative-strand RNA viruses, the templates for L polymerase-catalyzed replication and transcription of *Bunyaviridae* members are the ribonucleoproteins (RNPs) consisting of the full-length positive- or negative-strand RNA segments associated with the N protein.

To date, methods to study the role of *cis*-acting sequences at the 5′ and 3′ termini of viral RNA (vRNA) segments have been developed for Bunyamwera (BUN) virus (*Bunyavirus*) (7) and Rift Valley fever (RVF) virus (*Phlebovirus*) (24, 34), using the now classical T7-vaccinia virus (T7-VV) system (16) to express a chloramphenicol acetyltransferase (CAT) reporter cDNA flanked by 5′ and 3′ vRNA ends. An important step was taken when Bridgen and Elliott in 1996 (4) were able to rescue infectious BUN virus entirely from cloned cDNAs, although the procedure was cumbersome and the efficiency of generating infectious virus was rather low.

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TABLE 1. Oligonucleotide primers used to construct plasmids

Primer	Orientation	Sequence	Cloning product
RF9	Forward	AATCGTCTCT|AGGT*ACACAAAGACACGGCTACATG* ***Bsm*BI** *5′ UUK NTR (vRNA)*	pRF7 (UUK M) (vRNA)
RF2	Reverse	AATCGTCTCG|GGG*ACACAAAAGACGGCTACCATGGTA* ***Bsm*BI** *3′ UUK NTR (vRNA)* ***Nco*I** *G1 N-terminus*	
RF33	Forward	AATGAAGACGG|AGGT*ACACAAAGACGGCTACCATGGAGAA* ***Bbs*I** *5′ UUK NTR (cRNA)* *CAT N-terminus*	pRF19 (UUK M-CAT) (cRNA)
RF34	Reverse	AATGAAGACAT|GGGG*ACACAAAGACACGGCTACATG* ***Bbs*I** *3′ UUK NTR (cRNA)*	
RF10	Forward	AATGGGCC|*CTCTACGTCTGAGGC* ***Apa*I** *pol I promoter*	pRF20 (UUK M-CAT) (vRNA) + additional cleavage sites
RF30	Reverse	AATGAAGACTT|AGTCTAGAGCTCCCGGGATCCTCGA*GTCCCCCT*... ***Bbs*I** ***Xba*I** ***Sac*I** ***Sma*I** ***Bam*HI** ***Xho*I** *...ATTCCCCATCCC* *5′ UUK NTR (vRNA)*	
RF5	Forward	AATGGTCTCT|GACT*TAACGCCCCGCCCTGCCACT* ***Bsm*BI** *CAT C-terminus*	pRF33 (UUK M-CAT) (vRNA)
RF6	Reverse	AATCAATTGGGTCTCC|C*ATGGAGAAAAAAATCACTGG* ***Bsa*I** *CAT N-terminus*	
RF10	Forward	AATGGGCC|*CTCTACGTCTGAGGC* ***Apa*I** *pol I promoter*	pRF31 (UUK M-GFP) (vRNA)
RF4B	Reverse	AATGAAGACTT|*AGTCCCCCTATTCCCCATC* ***Bbs*I** *5′ UUK NTR (vRNA)*	
RF7B	Forward	AATCCCGGGAAGACTA|GACT*TAAGTGATGGTGATGGTG* ***Bbs*I** *GFP C-terminus*	
RF8	Reverse	AATCAATTGC|C*ATGGTGAGCAAGGGGCG* ***Nco*I** *GFP N-terminus*	
RF49	Oligolinker	CTAGTCAGTCAGTAGTCAGTCAGTG AGTCAGTCATCAGTGAGTGAGTCGATC ***Nhe*I compatible** Stop codons ***Nhe*I compatible**	pRF33 (UUK M-CAT) (vRNA)
RF50			

To look for an alternative approach for developing a reverse genetics system for *Bunyaviridae*, we have turned to the RNA polymerase I (pol I) expression system, which was recently successfully used to rescue infectious influenza virus (15, 31, 32). This system, originally developed by Hobom and coworkers (30, 49), has been used to study *cis*-acting sequences important for transcription and replication (14) and to develop a procedure for indirect selection of recombinant influenza viruses (12). An ambisense strategy to further simplify the procedure was recently reported (20). In the pol I system, cDNAs coding for viral RNA segments, or reporter genes flanked by viral sequences, are cloned between the RNA pol I promoter and terminator to generate transcripts that have correct 5′ and 3′ ends without modifications such as a cap structure and a poly(A) tail (12, 49). In the case of influenza virus, these pol I transcripts are then replicated and transcribed in the nucleus by the necessary viral proteins. Following transport of the RNPs to the cytoplasm, infectious particles are assembled by budding at the plasma membrane.

We have adopted the pol I system to express reporter genes flanked by the 5′ and 3′ noncoding sequences of the M RNA segment of Uukuniemi (UUK) virus, a member of the *Phlebovirus* genus (28). We have previously characterized extensively the molecular and cell biology of UUK virus. Full-length cDNAs corresponding to the L (6,423 nucleotides [nt]) (9), M (3,229 nt) (38), and S (1,720 nt) (42) segments have been constructed, and cDNAs encoding the open reading frames (ORFs) for the L, G1, G2, N, and NSs proteins have been derived (9, 26, 37, 44). As the first step toward the generation of infectious virus from cloned cDNAs, we show here that the pol I system can be used to synthesize chimeric RNA templates, which, despite lacking a cap structure and poly(A) tail, are transported to the cytoplasm, where they are amplified and transcribed by the UUK virus replicase components supplied either by superinfection with UUK virus or by expression of viral proteins from separate plasmids. The L and N proteins were found to be necessary and sufficient for transcription and replication, while NSs was completely dispensable. We also show that CAT activity could be transferred serially from culture to culture by passaging supernatants from transfected and superinfected cells. This indicates that the chimeric reporter RNA could be packaged into virus particles. Thus, the pol I system holds great potential as an effective alternative approach for a versatile reverse genetics system for members of the *Bunyaviridae* family.

MATERIALS AND METHODS

Cells and Virus. BHK-21 cells (American Type Culture Collection) were grown on plastic dishes in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS; Life Technologies, Gibco-BRL), 5%

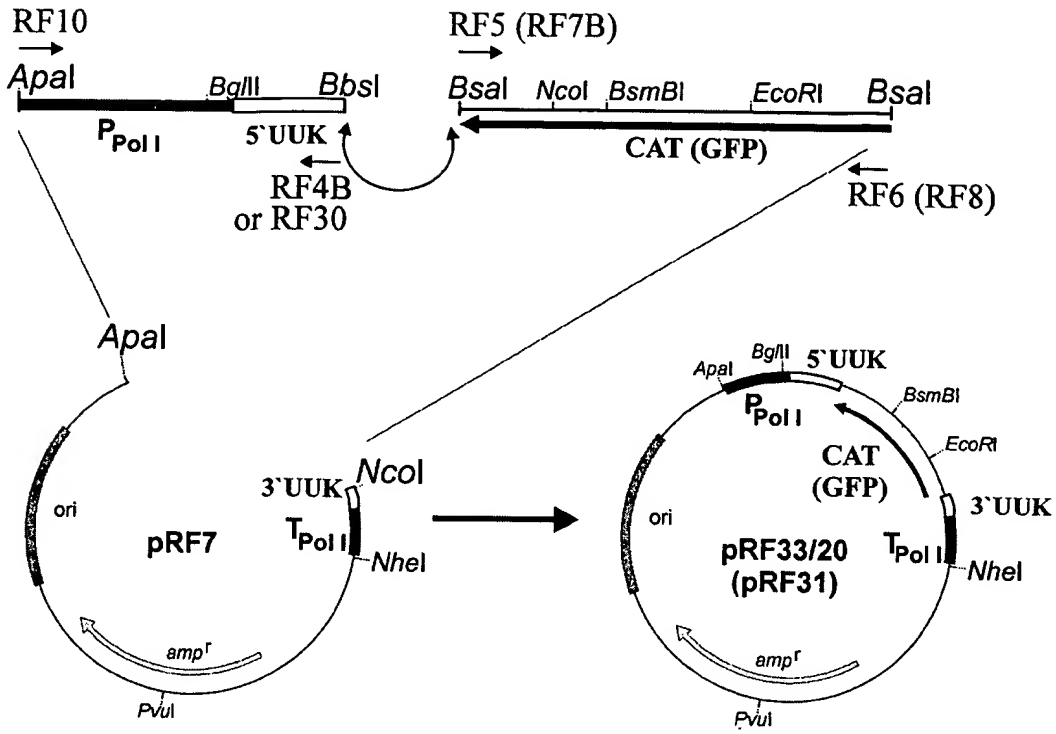


FIG. 1. Schematic diagram of the cloning strategy for constructing chimeric UUK virus reporter plasmids. Two PCR fragments, one containing the murine pol I promoter and the UUK M vRNA 5′ UTR (RF10/4B) and the other containing the CAT (RF5/6) or GFP (RF7B/8) ORF, were ligated with the large *ApaI-NcoI* fragment from plasmid pRF7 containing the UUK M vRNA 3′ UTR and the pol I terminator. This gave plasmids pRF33 (UUK M-CAT) and pRF31 (UUK M-GFP). Plasmid pRF20 was constructed by inserting multiple cloning sites immediately downstream of the UUK M vRNA 5′ UTR, using the two PCR fragments RF10/30 and RF5/6, respectively (see also Materials and Methods).

tryptose phosphate broth, 2 mM L-glutamine, 100 IU of penicillin/ml, and 100 μg of streptomycin/ml. The origin and the preparation of stock virus from the prototype strain S23 of UUK virus have been described elsewhere (33). The stock virus had a titer of 2×10^8 PFU/ml. Cells were infected with a multiplicity of infection (MOI) of about 5 to 10 PFU/cell.

Construction of plasmids. The PCR primers used for plasmid constructions are shown in Table 1. Plasmids designed for expression of UUK virus RNA molecules by RNA pol I *in vivo* carried the rRNA gene (rDNA) promoter region (−251 to −1 relative to the 45S pre-rRNA start point) and the rDNA terminator sequence (+571 to +745 relative to the 3′ end of the 28S rDNA) derived from murine rDNA (49). Between these two elements, UUK virus cDNA constructs from the M segment were exactly inserted in antisense orientation for vRNA expression (primer RF2/RF9) or in sense orientation for viral complementary (cRNA) expression (primer RF33/RF34). For efficient detection of protein expression following transcription and replication, the G1/G2 (p110) ORF in plasmid pRF7, encoding the full-length UUK virus M RNA (UUK M vRNA) segment, was replaced by reporter genes encoding CAT or a modified (enhanced) green fluorescent protein (GFP) (R. Flick and G. Hobom, unpublished data), using two PCR fragments (primers RF4B/RF10 and RF5/RF6, respectively) (Fig. 1; Table 1), without changing any of the nucleotides in the 5′ and 3′ untranslated regions (UTRs) of the UUK M segment. This resulted in pRF33 (UUK-M-CAT-vRNA), pRF31 (UUK-M-GFP-vRNA), or pRF19 (UUK-M-CAT-cRNA), respectively.

RNA pol I transcription plasmids were constructed using pHL1261 (12) or pRF42 (Fig. 2) as the basic vector system. After *Bsm*BI or *Bbs*I digestion, the vector plasmid can incorporate PCR fragments (*Bsm*BI or *Bbs*I restricted) in an oriented way. After transfection into different eukaryotic cell lines, the resulting constructs can be transcribed by RNA pol I, generating transcripts without any additional nucleotides or with modifications at the 5′ or 3′ end [e.g., cap structure or poly(A) tail].

Cloning constructs were verified either by dideoxy sequencing using an ABI PRISM 310 sequenator across the flanking regions and exchanging the central segment of the inserted PCR fragment by authentic (i.e., non-PCR-derived) DNA, using internally located unique restriction sites, or by sequencing across the entire PCR insert.

To insert the Mazon-Pfizer monkey virus (MPMV) constitutive transport element (CTE) sequence, additional cleavage sites (*XhoI-BamHI-SmaI/XmaI-SacI-XbaI*) were introduced exactly after the translation termination codon of the CAT gene in pRF33 using two PCR fragments (fragment 1, primer RF10/RF30; fragment 2, primer RF5/RF6) and the large *ApaI/NcoI* fragment from pRF7 (Fig. 1). The resulting construct pRF20 was used to insert two different forms of CTE sequences (MPMV 250 and MPMV 566; kindly provided by M.-L. Hammarskjöld, Charlottesville, Va.) in sense and antisense orientations (plasmids pRF35 to pRF38; see also Fig. 8).

For UUK virus protein expression, cDNAs from the UUK S segment encoding the N and NSs proteins, were inserted into the pcDNA3(+) vector (Invitrogen) using *Eco*RV cleavage. This gave rise to plasmids pCMV-UUK-N and pCMV-UUK-NSs. For UUK L expression, a *Not*I fragment from pBSK-UUK-L (kindly provided by R. Elliott, Glasgow, United Kingdom) was inserted into the *Not*I site of pcDNA3(+), giving rise to pCMV-UUK-L.

Transfection and superinfection with UUK virus. Plasmid DNA was transfected into subconfluent BHK-21 cells (3×10^6 to 6×10^6) using 2 to 4 μg of the respective plasmid and 8 to 20 μl of liposome plus buffer (LipofectAMINE PLUS; Life Technologies, Gibco-BRL) mixed in serum-free EMEM and incubated for 15 min at room temperature. After addition of 12 to 30 μl of liposome reagent, incubation was continued for a further 15 min. The cells were incubated at 37°C with the DNA-Lipofectamine mixture for 3 to 5 h. To determine the efficiency of transfection, plasmid pHL2823, expressing enhanced GFP (EGFP) under the cytomegalovirus (CMV) promoter (Flick and Hobom, unpublished), was transfected similarly. After further incubation for 20 h in EMEM containing 10% FCS and 5% tryptose phosphate broth, the transfected cells were washed with adsorption medium (EMEM supplemented with 20 mM HEPES and 0.2% bovine serum albumin) and superinfected with UUK virus at an MOI of 5 to 10 PFU/cell. After a 60-min adsorption period, the cells were washed once and incubated with adsorption medium for 15 to 30 h. A complete replication cycle takes place during this period.

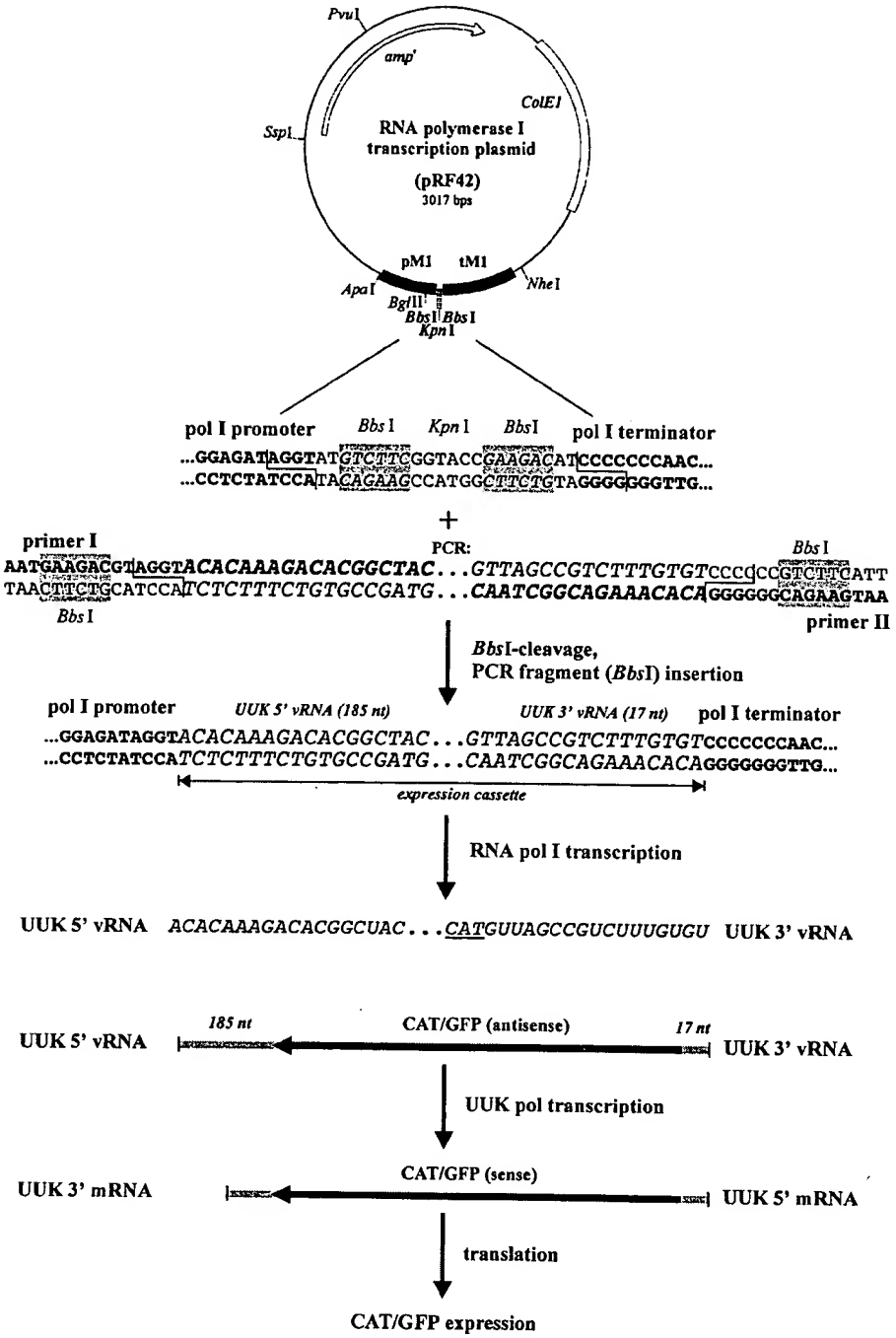


FIG. 2. Schematic diagram of the RNA polymerase I transcription plasmid (pRF42) and the generation of chimeric UUK virus-reporter RNA segments. To construct reporter plasmids, PCR-amplified expression cassettes are inserted between the *Bbs*I sites (shaded boxes) in the RNA pol I-driven expression plasmid pRF42. The cassettes are flanked by the murine RNA pol I promoter (pM1) and terminator (tM1) (shown in bold). The system allows for the transcription of any expression cassette by RNA pol I in rodent cell lines. The transcript starts exactly at the first position of the expression cassette and terminates at the last position before tM1, generating the correct 5′ and 3′ termini of the insert. *Bbs*I recognition sites are shaded; primers I and II used to amplify the expression cassette are shown in bold; UUK virus-specific 5′ and 3′ sequences are in italics. TAC, complementary to the ATG initiation codon in the reporter cDNA, is underlined.

CAT assay. Cell extracts were prepared as described by Gorman et al. (17). In an initial series, 50 μl of each cell lysate (prepared from 10^6 cells in the case of cotransfection experiments or from 3×10^6 cells in the case of superinfection experiments), and depending on the results, serially diluted samples of the various cell lysates were mixed with 10 μl of acetyl coenzyme A (4 mM) and 10 μl of fluorescence-labeled chloramphenicol (boron dipyrromethane difluoride fluorophore substrate; Flash Cat kit; Stratagene) and incubated at 37°C for 2 h. For extraction of reaction products, 0.5 ml of ethylacetate was added; after centrifugation for 1 min at 15,000 × *g*, the upper phase containing the reaction products was isolated and the solvent was evaporated. The resulting pellet was resuspended in 20 μl of ethylacetate, and the reaction products were separated by thin-layer chromatography (TLC plates, 20 by 20 cm; Silica Gel 60; Merck)

using a solvent mixture (mobile phase) of chloroform and methanol (87:13). Finally, the reaction products were visualized by UV illumination, documented by photography, and evaluated using WinCam software (Cybertech, Berlin, Germany) or Quantity One (Bio-Rad). Ratios of activities were calculated based on at least three independent sets of serial dilutions of cell lysates down to a level of 30 to 50% product formation.

Indirect immunofluorescence microscopy. BHK-21 cells grown on coverslips were transiently transfected as described above. Five to eight hours after transfection, the cells were washed with phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde in PBS for 15 min at room temperature, washed with PBS again, quenched with 10 mM glycine for 20 min at room temperature, and finally washed with PBS. Depending on the antibodies used, the cells were permeabilized either with 0.1% Triton X-100 for 30 min or with methanol for 2 min at room temperature. Cells were incubated with PBS containing 0.1% bovine serum albumin and incubated for 30 min with a monoclonal antibody against the N protein (J. Veijola, A. Bergström, and R. F. Pettersson, unpublished data) and a rabbit polyclonal antiserum against NSs (44). Primary antibodies were visualized with tetramethyl rhodamine isothiocyanate-conjugated anti-mouse immunoglobulin G or fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G secondary antibody, washed, and mounted in 50% glycerol containing 50 mM Tris-HCl (pH 8.0) and 9.2 mM *p*-phenylenediamine.

To visualize GFP expression, coverslips were immersed in PBS instead of the standard mounting solution, and fluorescence was monitored at 48 h after transfection with expression plasmids pHL2823 (CMV-GFP) and pRF31 (UUK M-GFP). Immunofluorescence micrographs were obtained either with an Axiophot (Zeiss) or an Eclipse E1000M (Nikon) fluorescence microscope. The latter was equipped with a Spot charge-coupled device camera (Diagnostic Instruments, Inc.).

Serial passaging of virus-containing supernatants. BHK-21 cells were cotransfected with plasmid pRF33 (UUK M-CAT vRNA), together with the L (pCMV-UUK-L) and N (pCMV-UUK-N) expression plasmids, followed by superinfection with UUK virus at an MOI of 5 to 10 PFU/cell 20 h later. Cells were analyzed for CAT activity 30 h postinfection, and the corresponding supernatants were used for virus passaging. Cell debris was removed by centrifugation at 10,000 rpm (13,000 × *g*) for 5 min; a 2-ml sample of undiluted supernatant was used to infect a dish containing 6×10^6 BHK-21 cells and incubated for 60 min. After a change of medium (EMEM, 10% FCS) and incubation for 24 to 30 h, cells and supernatants were treated and passaged another round as described above.

RESULTS

General strategy of the pol I-driven expression system. Our strategy to develop a reverse genetics system for UUK virus by using the RNA pol I expression system basically followed the one established for influenza virus (12, 13, 14, 30, 31, 32, 49). Reporter cDNAs containing the exact ORF for either CAT or GFP (in antisense orientation) were flanked either by the 5′ (185-nt) and 3′ (17-nt) ends of the M vRNA segment or by the cRNA ends (17 and 185 residues, respectively; only for CAT) (Fig. 1 and 2) (38). The CAT and GFP ORFs exactly replaced that of p110, the precursor of G1 and G2 (38, 46), such that no extra nucleotides or any other changes were introduced in the 5′ or 3′ UTR. These chimeric cDNAs were then cloned into plasmid pRF42 (Fig. 2) or pHL1261 (12) between the promoter and terminator of the murine rDNA gene to generate plasmids pRF33 (expressing CAT-M vRNA), pRF31 (GFP-M vRNA) (Fig. 1), and pRF19 (CAT-M cRNA).

In initial control experiments using the pol I CAT-M vRNA construct, we observed a weak CAT activity in the absence of any viral proteins provided from expression plasmids or superinfection. Analysis of the nucleotide sequence upstream of the CAT ORF (i.e., within and beyond of the pol I terminator [Fig. 1]) revealed an AUG codon in frame with the CAT initiator AUG with no intervening in-frame stop codon. We assumed that a weak cryptic promoter upstream of the AUG was generating a transcript that could be translated into an active CAT enzyme. We therefore introduced an oligonucleotide encoding translational stop codons in all three ORFs in the *Nhe*I site (Fig. 1; Table 1) between the two AUGs. This completely abolished the background CAT activity. This modification was introduced in the three plasmids pRF19, pRF31, and pRF33.

Based on the results from the influenza system, pol I-driven transcription of the reporter RNAs will initiate and terminate exactly at the 5′ and 3′ of the inserted cDNAs, thus giving rise to transcripts with the correct vRNA or cRNA ends (12, 49). The nucleotide sequences and the strategy for the synthesis of the vRNA sense transcript are shown in Fig. 1 and 2. Following transport to the cytoplasm, these pol I transcripts would be transcribed and replicated by the necessary viral proteins either expressed from plasmids encoding the individual proteins or provided by superinfection with UUK virus. For the synthesis of viral proteins, plasmids expressing mRNAs for L (pCMV-UUK-L), N (pCMV-UUK-N), or NSs (pCMV-UUK-NSs) under the CMV promoter were constructed from previously cloned cDNAs containing the corresponding ORFs. Immunofluorescence analyses of transfected cells showed that N and NSs were efficiently synthesized (data not shown).

Expression of CAT and GFP from chimeric cDNAs flanked by the UUK M vRNA or cRNA 5′ and 3′ ends. The reporter plasmid pRF33 (CAT-M vRNA) or pRF19 (CAT-M cRNA) was introduced into BHK-21 cells by liposome-mediated transfection. Control experiments using a CMV-EGFP expression plasmid pHL2823 (Flick and Hobom, unpublished) indicated a transfection efficiency of about 20 to 25% (see Fig. 4A). To drive replication and transcription of the chimeric RNA, cells were either infected with UUK virus 20 to 24 h after transfection or cotransfected with expression plasmids encoding L, N, and NSs. Some cultures were both cotransfected with the expression plasmids and superinfected with virus. As shown in Fig. 3, CAT activity was readily detected in lysates from cells transfected with pRF33 and superinfected with UUK virus (lane 2). Interestingly, CAT activity was about 2.4-fold stronger in the lysate from pRF33-transfected cells expressing L, N, and NSs from the plasmids (lane 3) than in that from superinfected cells (activity arbitrarily set at 100%). Superinfection combined with the expression plasmids did not further enhance CAT activity (lane 4).

Very weak CAT activity was observed in cells transfected with pRF19 (CAT-M cRNA) and infected with UUK virus (Fig. 3, lane 6), while strong activity was evident in cells cotransfected with the plasmids expressing the three viral proteins (lane 7). Again, no enhanced effect was seen if infection was combined with transfection of expression plasmids (lane 8). In the absence of viral superinfection or expression plasmids, no CAT activity was observed in either case (lanes 1 and 5), showing that regardless of whether synthesis is in sense or antisense orientation, the pol I transcript cannot be directly translated into CAT (see also Materials and Methods).

About 5 to 10% of BHK-21 cells transfected with pRF31 (GFP-M vRNA) and cotransfected with expression plasmids were GFP positive (Fig. 4C). The intensity varied between cells, ranging from weak to intense. No GFP-positive cells were observed in the absence of expression plasmids (Fig. 4E). As mentioned above, about 20 to 25% of the cells were positive when GFP was expressed from a plasmid under the CMV promoter (Fig. 4A).

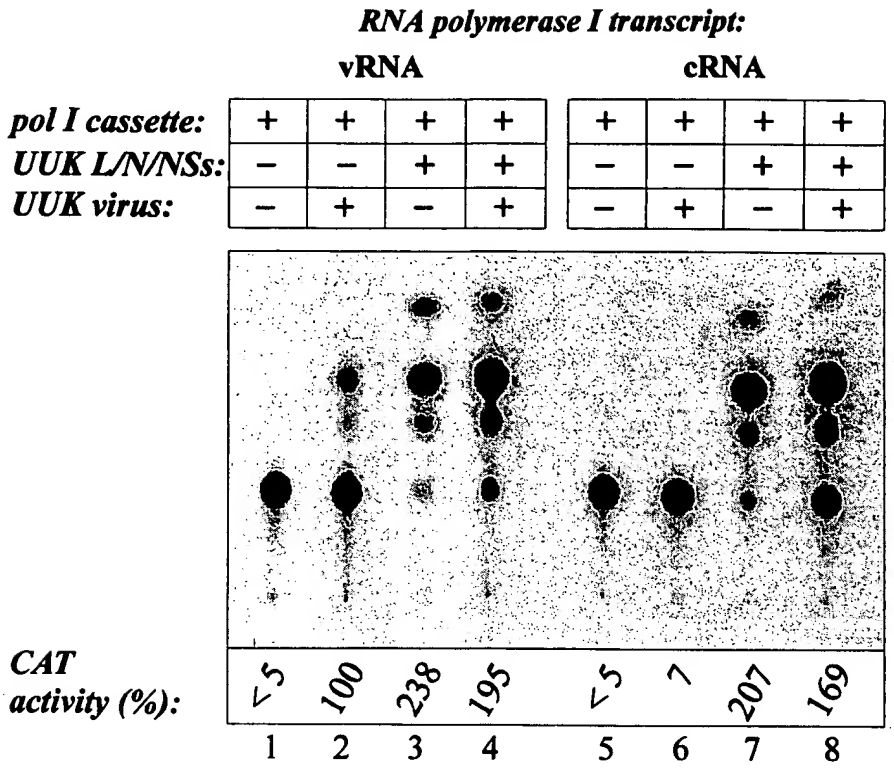


FIG. 3. CAT activity in BHK-21 cells transfected with pRF33 or pRF19 expressing UUK M-CAT RNA chimeras is dependent on UUK virus proteins. BHK-21 cells were transfected with plasmids pRF33, producing vRNA sense transcripts (lanes 1 to 4), or pRF19, producing cRNA sense transcripts (lanes 5 to 8). Viral helper proteins were supplied either by cotransfection with plasmids expressing the L (pCMV-UUK-L), N (pCMV-UUK-N), and NSs (pCMV-UUK-NSs) proteins (lanes 3 and 7), by superinfection with UUK virus 24 h posttransfection (lanes 2 and 6), or by the combination of superinfection and protein expression (lanes 4 and 8). CAT activity was assayed 20 h postinfection (lanes 2 and 6), 20 h posttransfection (lanes 3 and 7), or 44 h posttransfection (lanes 4 and 8). As shown by transfection with the reporter plasmids alone (lanes 1 and 5), CAT activity was found to be completely dependent on viral protein expression. CAT activity measured from each lysate is expressed as percentage of the activity obtained from superinfected cells, which was arbitrarily set at 100 (lane 2).

Taken together, these results indicated that pol I-transcribed reporter RNAs are transported to the cytoplasm, where they are transcribed and most likely also replicated by the viral polymerase components to yield the desired reporter protein.

Optimization of reporter gene expression. To find the optimal conditions for reporter expression, cells were transfected with different ratios of the L, N, and NSs expression plasmids. As shown in Fig. 5A, the NSs protein was found to be completely dispensable for CAT expression, since the same level of activity was observed without NSs and in the presence of increasing amounts of NSs plasmids. The importance of the relative levels of L and N expression was also analyzed. No activity was observed in the absence of the L (Fig. 5B, control lane) or N (Fig. 5C, control lane) plasmid. As shown in Fig. 5B and C, the molar ratio between the L and N expression plasmids was not critical. Molar N:L plasmid ratios of 8:1 (Fig. 5B) or molar L:N plasmid ratio of 8:1 (Fig. 5C) yielded similar levels of CAT activity. Since NSs was not required for CAT expression, plasmid pCMV-UUK-NSs was omitted from the expression studies described below.

We next analyzed whether the timing of the transfection of the reporter plasmid relative to transfection of the L and N expression plasmids (Fig. 6), or virus superinfection (Fig. 7), was critical for optimal expression. The protocols used for the plasmid transfections are outlined in Fig. 6A. Cells were transfected simultaneously with the reporter and expression plasmids (transfection I), or the expression plasmids were transfected 6 h before the reporter plasmid (transfections IIa and IIb), or vice versa (transfections IIIa and IIIb). Cell lysates were then analyzed for CAT activity at various time points after transfection (Fig. 6B to D). The highest CAT activity was recorded in cells at 24 to 48 h following cotransfection with all three plasmids (Fig. 6B). Somewhat lower activity was also observed at 48 h if the two transfections were separated by 6 h (Fig. 6C and D). It should be noted that the time points above the CAT signals in Fig. 6C and D indicate the time after the second transfection (see also the legend to Fig. 6).

Figure 7A shows the protocol used for reporter plasmid transfection followed by superinfection with UUK virus. The reporter plasmid was transfected into cells 32 (transfection I), 24 (II), or 8 (III) h before superinfection, followed by analysis of CAT activity from cell lysates prepared at 16, 24, and 32 h postinfection. The highest activity was observed if transfection preceded superinfection by 32 h (Fig. 7B), and the weakest was seen if the superinfection was done 8 h after transfection (Fig. 7D). Intermediate activity was observed for the transfection II protocol (Fig. 7C).

Based on these analyses, we have adopted the following standard conditions for maximum reporter expression: (i) a molar ratio of L and N plasmids of 2:1, (ii) cotransfection of

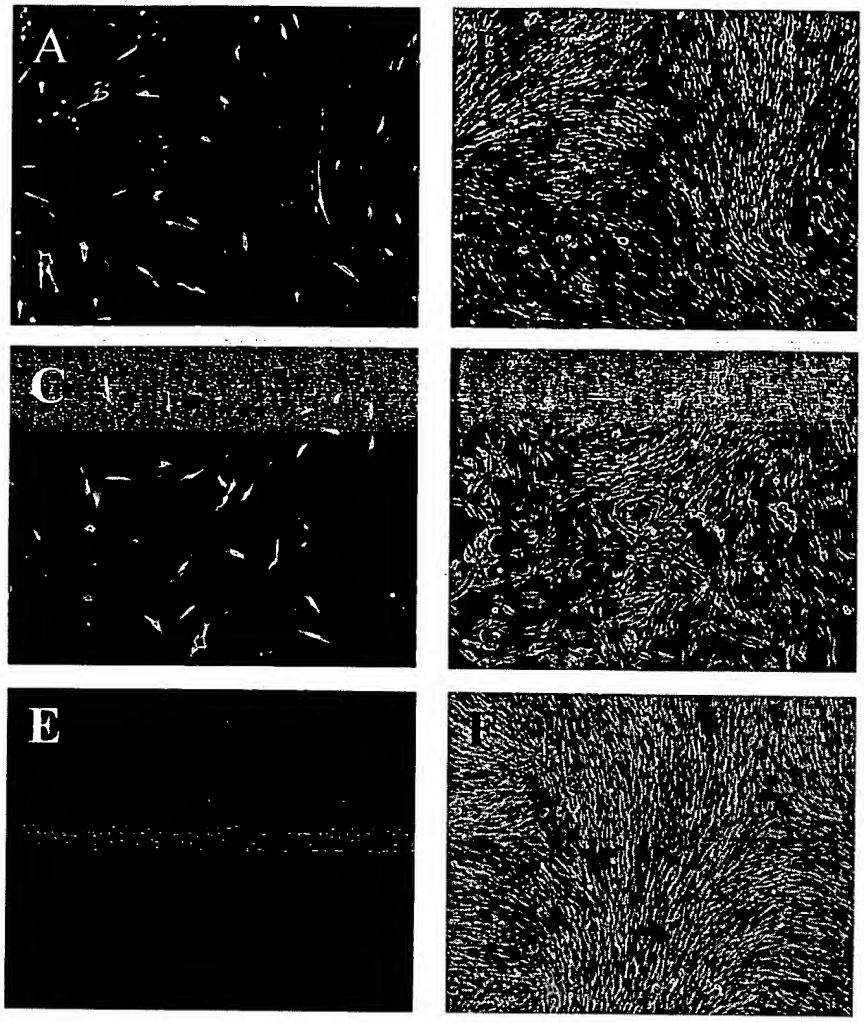


FIG. 4. Immunofluorescence analysis showing that expression of GFP from pRF31 is dependent on UUK virus proteins. (A) BHK-21 cells transfected with only pHL2823 encoding GFP under the control of the CMV promoter; (B) same cells as in panel A, viewed by regular light microscopy; (C) cells cotransfected with the reporter plasmid pRF31 (UUK M-GFP vRNA) and the expression plasmids pCMV-UUK-L and pCMV-UUK-N; (D) same cells as in panel C, viewed by light microscopy; (E) cells transfected with pRF31 alone, with GFP expression visualized 48 h later by fluorescence microscopy; (F) same cells as in panel E, viewed by light miccropscopy.

the pol I cassette (reporter) plasmid and the L and N expression plasmids followed by CAT assay 24 h later, and (iii) superinfection with UUK virus 20 to 24 h after transfection with the pol I cassette plasmid followed by CAT assay 24 h later.

Effect of CTE sequences on CAT expression. Pol I transcripts lack a cap structure and a poly(A) tail. This is also true for the chimeric reporter transcripts (12, 49). We initially worried that these RNA species would be inefficiently exported from the nucleus to the cytoplasm. To analyze if nuclear export could be further enhanced, we first inserted additional restriction enzyme cleavage sites (Table 1) into pRF33 (UUK M-CAT) exactly between the CAT translation termination codon and the UUK M segment 5′ UTR, generating plasmid pRF20 (Fig. 1). We then inserted a longer (MPMV 566) and a shorter (MPMV 250) version of the CTE sequence from MPMV into pRF20 (Fig. 8). This CTE sequence has been shown to greatly enhance export of the intron-containing MPMV RNA from the nucleus (3, 11). The CTE sequences were inserted in either the sense or the antisense orientation (Fig. 8A). The pol I expression cassette and the L and N expression plasmids were cotransfected into BHK-21 cells, and cell lysates were analyzed for CAT activity 24 h later. As seen in Fig. 8B, the CTE sequence in either orientation had no enhancing effect on the reporter gene activity (lanes 4 to 7). The CTE in the antisense orientation had a slight inhibitory effect on CAT activity (lanes 6 and 7), possibly because the stem-loop structure in this orientation constitutes a relative physical barrier for the RNA polymerase; alternatively, the sequence context in the antisense orientation could interfere with replication or transcription signals in the noncoding region of the UUK virus segment. The results also showed that the additional restriction enzyme cleavage sites introduced into pRF20 had no disturbing effect on CAT expression compared to pRF33 (lanes 2 and 3).

Generation of recombinant UUK virus. Finally, we analyzed whether the chimeric reporter RNA could be packaged into infectious virus particles that could be serially passaged to fresh cultures. BHK-21 cells were either cotransfected with the

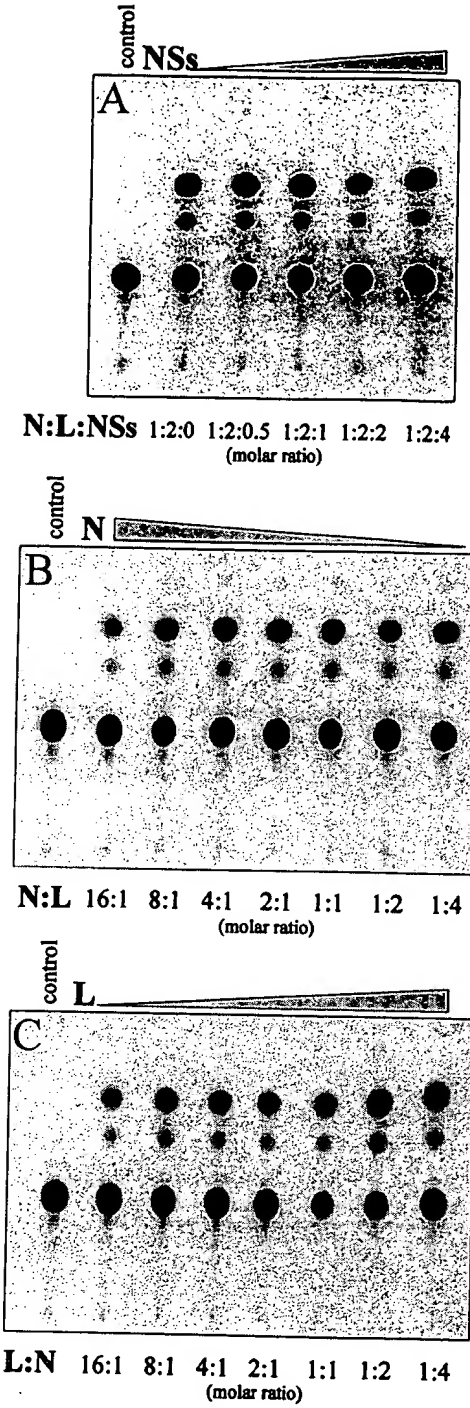


FIG. 5. Optimization of reporter gene expression by titration of UUK L, N, and NSs expression plasmids. (A) Titration of pCMV-UUK-NSs. BHK-21 cells (3×10^6) were cotransfected with constant amounts of the reporter plasmid pRF33 (UUK M-CAT vRNA) (1 μg), expression plasmids pCMV-UUK-L and pCMV-UUK-N in a molar ratio of 2:1, and various amounts of pCMV-UUK-NSs as indicated. A sample corresponding to 1/50 of the cell lysate prepared at 20 h posttransfection was used for CAT reactions. In the control experiment (leftmost lane), BHK-21 cells were transfected only with pRF33 (1 μg). (B) Titration of pCMV-UUK-N. BHK-21 cells were transfected with pRF33 (1 μg) and a constant amount of pCMV-UUK-L (2.5 μg) together with various molar amounts of pCMV-UUK-N as indicated. CAT activity was determined as for panel A. (C) Titration of pCMV-UUK-L. BHK-21 cells were transfected with pRF33 (1 μg) and a constant amount of pCMV-UUK-N (0.3 μg) together with various molar amounts of CMV-UUK-L as indicated. To achieve equal transfection conditions in each experiment, the total transfected DNA was adjusted to the same level by adding plasmid pHL2823 (CMV-GFP).

reporter plasmid pRF33 (CAT-M vRNA) and the L and N expression plasmids or transfected with the reporter plasmid alone, followed in both cases by superinfection with UUK virus 20 h later. The medium was collected 30 h later (i.e., 50 h posttransfection) and used to infect new BHK-21 cultures. This was repeated for another cycle. From each passage, cell lysates were analyzed for CAT activity. As shown in Fig. 9, CAT activity was strong in cells transfected with the combination of the three plasmids and infected with UUK virus (lane 1). CAT activity could be passaged at least twice, although the activity was reduced between each passage (lanes 1 to 3). If superinfection was omitted, no CAT activity could be detected upon passaging (data not shown), nor could CAT activity be passaged if cells were transfected only with the reporter plasmid followed by superinfection (lanes 4 to 6).

DISCUSSION

In this study, we have successfully adopted the RNA pol I transcription system (30, 31, 49) for the development of a reverse genetics protocol for members of the *Bunyaviridae* family. As a model virus, we used UUK virus, a member of the *Phlebovirus* genus. Reporter cDNAs encoding CAT or GFP flanked by the terminal sequences of the UUK M RNA segment were transcribed in the cell nucleus by pol I, transported to the cytoplasm, and transcribed and amplified by the RNA polymerase L in the presence of the nucleoprotein N provided either from expression plasmids or by superinfection with UUK virus. Our results indicate that expression of the L and N proteins alone yielded more efficient reporter expression than could be achieved by superinfection and that the nonstructural NSs protein was completely dispensable for CAT activity. Finally, CAT activity could be serially passaged from culture to culture, indicating that the chimeric reporter RNA was packaged into virions.

We decided to use the 5′ and 3′ untranslated sequences from the M RNA segment to flank the reporter ORFs, rather than those from the L or S segment. In virus-infected BHK-21 cells, the M RNA segment is synthesized in a twofold molar excess over the L RNA and a fourfold excess over the S RNA (33), suggesting that the M RNA may possess the strongest promoter. In addition, the S RNA is transcribed into two subsegmental mRNAs encoding N and NSs using an ambisense strategy (42), which might complicate the use of the terminal sequences from this RNA segment. Our previous results have indicated that the mRNA transcribed from the M segment and translated into p110, the G1/G2 precursor, is about 100 nt shorter than the M vRNA template (R. Rönnholm and R. Pettersson, unpublished data) due to an as yet uncharacterized transcriptional termination signal. To ensure proper transcription termination of the reporter mRNAs, we therefore included the whole 185-residue-long UTR from the 5′ end of the vRNA (or 3′ end of the cRNA, depending on the

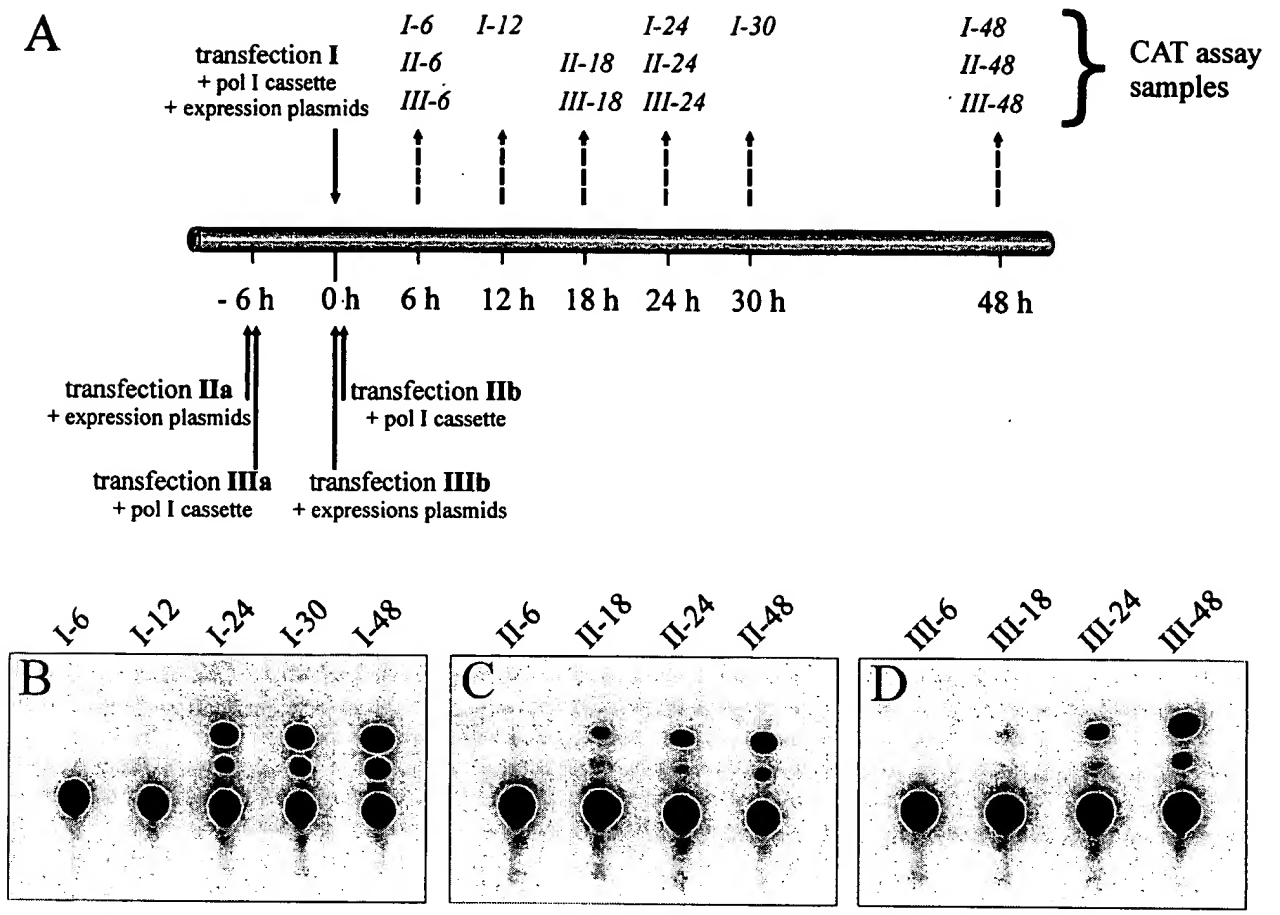


FIG. 6. Optimization of time intervals between transfection with reporter plasmid and viral protein-expressing plasmids, and analysis of CAT activity. (A) Schematic diagram depicting the timetable for transfection with the RNA pol I expression cassette plasmid (pRF33) and the viral expression plasmids (pCMV-UUK-L and pCMV-UUK-N) (solid arrows), as well as the time points for assaying CAT activity (broken arrows). Plasmids were either cotransfected (I) or transfected in either order separated by 6 h (II and III). CAT activity was assayed at indicated times after the last transfection (set as time zero). (B to D) Analysis of CAT activity from the transfection protocols shown in panel A. To be able to determine minor differences in expression levels, cell lysates were diluted 1:50. The number of the experiment (I to III) and time points (hours) when cell lysates were harvested are shown above the CAT signals.

polarity of the construct). Previous work with BUN (7), and RVF (34) viruses has shown that the extreme 3′ end contains sequence elements critical for transcription initiation.

The fact that pol I reporter transcripts are noncapped and nonpolyadenylated raised the concern that these RNAs would not be efficiently transported out of the nucleus. In the case of influenza virus, the pol I transcripts do not have to exit the nucleus, since transcription and replication of vRNAs take place in the nucleus. In contrast, *Bunyaviridae* members replicate solely in the cytoplasm and the pol I transcripts therefore have to be exported from the nucleus. Our results showed that these concerns were unfounded, since CAT and GFP activities were readily detected. Newly synthesized nuclear RNA species rapidly associate with a set of proteins to form RNP structures. Some of these proteins contain an export signal and serve as export factors that guide the RNPs to and through the nuclear pore complex (29). We can only speculate that such an export factor(s) binds to our chimeric reporter RNA and facilitates its export to the cytoplasm. The CTE of MPMV has been shown to stimulate the export of unspliced RNAs from the nucleus (3, 11). To analyze whether the CTE could further enhance the export of pol I transcripts as assayed by increased reporter activity, we introduced two CTE variants in the sense or antisense orientation downstream of the 5′ UTR of the CAT-M vRNA. These constructs did not enhance CAT expression, suggesting either that the CTE does not work in this sequence context or that the RNA export is already high enough to allow amplification of the reporter RNA by the viral proteins.

The finding that expression of the L and N proteins was more efficient compared to superinfection in stimulating CAT activity is interesting. One can only speculate as to the reason for this result. For all negative-strand RNA viruses, the template for transcription and replication is an RNP made up of the vRNA and the nucleocapsid protein. The formation of such RNPs is thus a prerequisite for the polymerase to transcribe and replicate template RNAs. It is conceivable that in virus-infected cells, transcription, replication, and viral protein synthesis are compartmentalized in some kind of "virus factories" to which the pol I transcripts might not readily have access. Thus, the pol I transcripts would have a rather low

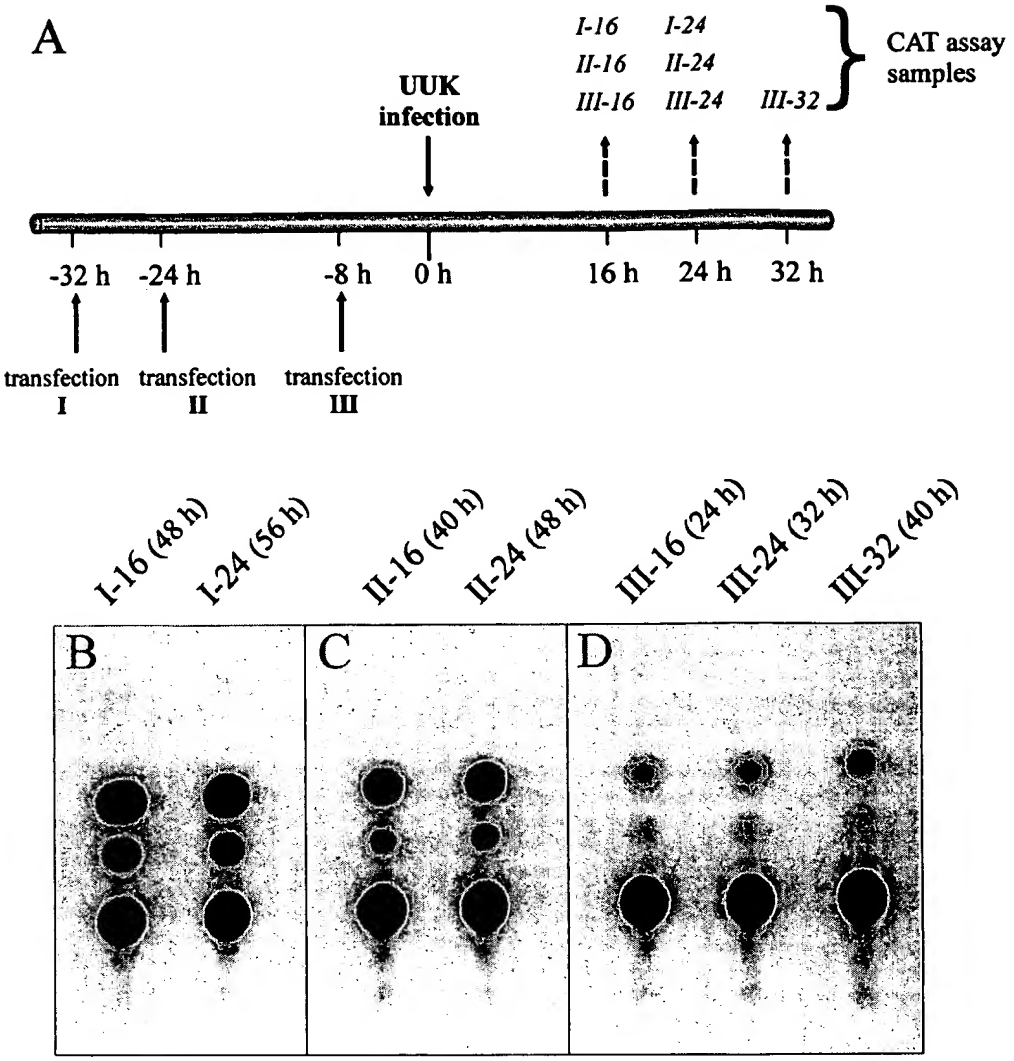
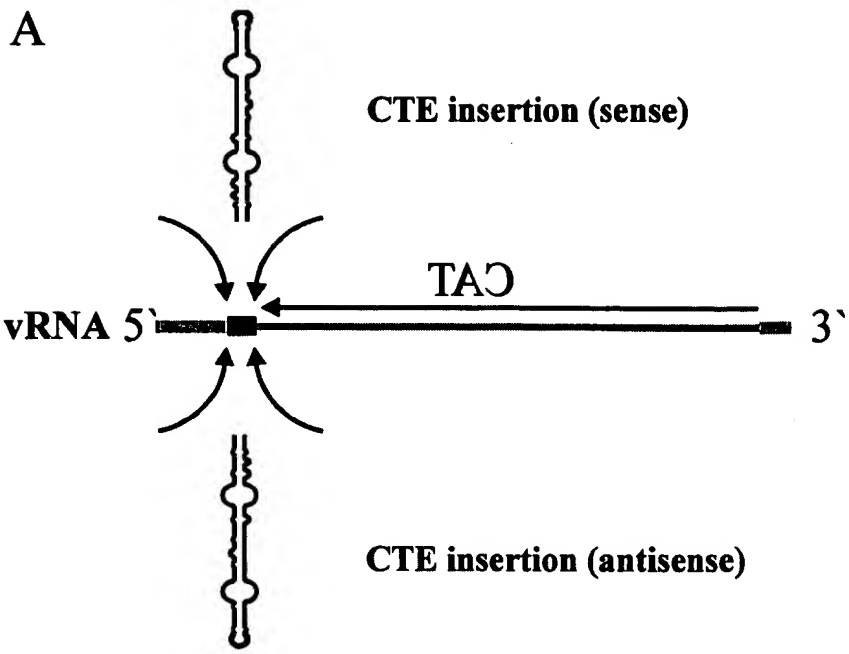


FIG. 7. Optimization of time intervals between plasmid transfections, superinfection with UUK virus, and analysis of CAT activity. (A) Schematic diagram depicting the timetable for (i) cotransfection with the RNA pol I expression cassette plasmid (pRF33) and the two plasmids expressing L and N, (ii) superinfection with UUK virus, and (iii) CAT assay. Plasmids were cotransfected either 32 (I), 24 (II), or 8 (III) h before superinfection (set as time zero). (B to D) Analysis of CAT activity from the transfection/superinfection protocols shown in panel A. To be able to determine minor differences in expression levels, cell lysates were diluted 1:50. The number of the experiment (I to III) and time points when cell lysates were harvested are shown above the CAT signals. The time period between transfection and CAT assay is shown in brackets.

probability of being encapsidated by the N protein. In contrast, newly synthesized virion-derived RNAs would immediately associate with newly synthesized N protein and thus serve as templates for further replication and transcription. In L- and N-expressing cells, such competition and spatial constraints would not exist, resulting in efficient transcription and replication of the pol I transcripts. In addition, it is also likely that rather few pol I transcripts reach the cytoplasm and that they are simply outcompeted by the much more efficient vRNA synthesis.

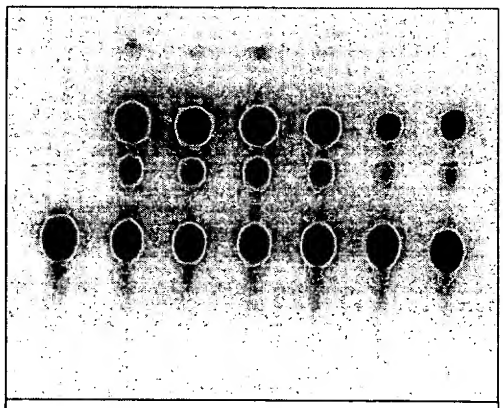
In the experiments described here, we used the murine pol I promoter/terminator sequences to express the reporter constructs in BHK-21 cells. Although a transfection rate of 20 to 25% was regularly achieved, the use of the human pol I promoter and highly transfectable human embryonic kidney cells (293T) (20, 31, 32) has the potential to further increase the efficiency of reporter expression.

The function of the S RNA segment-derived NSs protein has remained elusive. We found that NSs was not required for CAT activity. Previous work with a natural mutant of RVF virus (clone 13), which has a large internal in-frame deletion in the NSs gene, has shown that it replicates normally in some cell lines while establishing abortive infections in others, and that it is avirulent in mice and hamsters (27, 47). Using an in vitro transcription-replication system, it was recently shown that NSs of RVF virus had neither a stimulatory nor an inhibitory effect on transcription (24, 34). Finally, NSs has similarly been shown not to be required for the transcription of BUN virus RNAs (7). Recently, an NSs deletion mutant of BUN virus was made by using a reverse genetics system (A. Bridgen, J. K. Fazaker-



B

pol I cassette:	pRF33	pRF33	pRF20	pRF36	pRF37	pRF35	pRF38
MPMV:	/	/	/	566	250	566	250
orientation:	/	/	/	*sense*	*sense*	*anti-sense*	*anti-sense*
UUK L/N:	−	+	+	+	+	+	+



CAT activity (%):	<5	100	97	101	90	45	58
	1	2	3	4	5	6	7

FIG. 8. The CTE from MPMV RNA does not increase CAT activity expressed from reporter plasmids. (A) Schematic diagram showing the insertion of one of two forms (MPMV 566 or MPMV 250) of the CTE (in either sense or antisense orientation) between the 5′ UTR of the UUK M vRNA and the CAT ORF. (B) CAT activity in lysates from BHK-21 cells cotransfected with the CTE-containing reporter plasmids and the UUK L and N expression plasmids. Lane 1, CAT activity in cells transfected without viral expression plasmids. CAT activity in cells cotransfected with pRF33 (CAT-M vRNA) and UUK L and N expression plasmids was arbitrarily set at 100 (lane 2). Plasmid pRF20, containing an additional multiple cloning site downstream of the UUK 5′ UTR, displayed the same CAT activity as the parental pRF33 plasmid. CAT activity measured from the other lysates is expressed as percentage of that obtained in this control.

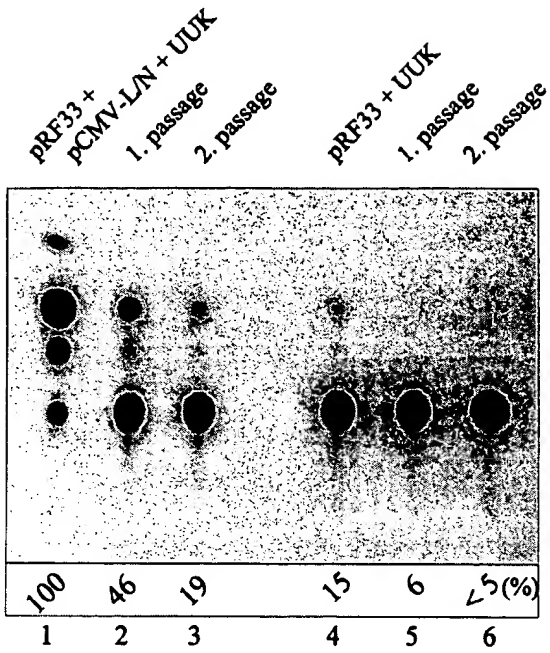


FIG. 9. Analysis of CAT activity in BHK-21 cells after serial passages of supernatants containing recombinant UUK virus. BHK-21 cells were cotransfected either with pRF33 and UUK L and N expression plasmids (lane 1) or only with pRF33 (lane 4) 24 h prior to superinfection with UUK virus (MOI of 10). Cell lysates were prepared 30 h later and assayed for CAT activity, while the media were collected and undiluted samples were transferred to new BHK-21 cells. This was repeated for another cycle, and CAT activity was assayed after each passage. CAT activities are expressed as percentage of the activity obtained after the first transfection/superinfection (lane 1).

ley, and R. M. Elliott, Abstr. XIth Int. Congr. Virol., abstr. VW47.06, 1999). This mutant grew to somewhat lower titers than wild-type virus, had a small-plaque phenotype, displayed a reduced shutoff of host cell protein synthesis, and had an attenuated pathogenicity when inoculated into mice. Our results reported here are thus in conformity with these results showing that NSs is not essential for transcription or replication in tissue culture cells.

One important question in regard to our results is whether the pol I transcript is amplified by replication. Although we have not directly quantified RNA synthesis, we argue that the observed high expression level of CAT and GFP could not have been achieved unless replication had occurred. Immunofluorescence analysis showed that individual cells displayed a very strong GFP signal. Based on our previous experience (13, 14), the overall level of CAT activity was much higher than that obtained in the influenza virus pol I system, even compared to the most effective influenza virus up-regulation mutant. Finally, the fact that extracellular medium from transfected and UUK virus-superinfected cells could be used to serially passage CAT activity strongly suggests that the pol I transcript must have been amplified and packaged.

A reverse genetics system was developed some years ago for BUN virus (4, 7). Using the T7-VV RNA polymerase-driven system, full-length L, M, and S antigenome RNA segments were expressed from plasmids under the T7 RNA polymerase promoter. Correct 3′ ends were generated with the hepatitis δ ribozyme. All viral proteins were expressed under the T7 promoter from plasmids encoding L, G1, G2, NSm, N, and NSs. This is to date the only system that has allowed the rescue of an infectious *Bunyaviridae* member entirely from cloned cDNAs without the use of the homologous helper virus. Although constituting a major advance in *Bunyaviridae* research, this system still suffers from low efficiency and the need to use the VV helper to drive expression.

A reverse genetics system has also been developed for RVF virus, which like UUK virus is a phlebovirus (24, 34). In this system, the antisense CAT reporter cDNA was expressed using the T7-VV system, while the L and N proteins were supplied from VV recombinants. No recombinant RVF virus has been reported to have been produced using this system.

Arenaviruses also contain a segmented (bipartite), negative-strand genome (39). Recently, the first report on the development of a reverse genetics system for lymphocytic choriomeningitis virus was published (23). Although no recombinant virus was rescued, it was shown that only the RNA polymerase (L) and nucleoprotein (NP) proteins were sufficient to support transcription and replication of a CAT reporter flanked by 5′- and 3′-terminal viral sequences. Both the reporter transcript and the viral proteins were expressed by using the T7-VV system.

The pol I system offers clear advantages over the VV-based reverse genetics systems used for many other negative-strand viruses. VV has been used either to direct the synthesis of the T7 RNA polymerase (16), which then drives the expression of the reporter construct and the viral proteins (1, 5, 18, 19, 22, 23, 41, 48), or to express the viral helper proteins directly (7, 24, 34). VV introduces into the cell a number of unwanted enzymatic activities, which are avoided by using the pol I system. In addition, there is no need to remove the VV, by physical or biochemical means (22, 41, 48), by passaging the virus through cells not permissive to VV (4) or by using a variant VV (MVA-T7) which does not replicate in mammalian cells (45). The pol I system also has the advantage of generating the exact 5′ and 3′ ends of the RNA transcripts, thus avoiding the need for expressing runoff transcripts from restriction enzyme-cleaved plasmids or the use of a ribozyme to produce the correct 3′ end.

The pol I system has recently been successfully developed to reconstitute infectious influenza virus entirely from cloned cDNAs (15, 20, 31, 32). Our present results suggest that this could also be possible for *Bunyaviridae* members. In analogy to the influenza virus protocol, all three full-length RNA segments would be expressed from the pol I promoter, preferably as antigenomes (positive strands) (4, 22, 41), while the L, G1, G2, N, and possibly also the NSs proteins would be expressed from plasmids using the CMV promoter. Such a system would allow further characterization of *cis*- and *trans*-acting determinants important for the regulation of transcription and replication, as well as for virus maturation and packaging. It might also allow for the generation of genetically altered recombinant viruses to study structure-function relationships and molecular aspects of viral pathogenicity and the engineering of effective attenuated vaccines. It also remains to be investigated whether this approach is generally applicable to *Bunyaviridae* members other than UUK virus, such as the medically important hanta- and nairoviruses.

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Mutational Analysis of the Uukuniemi Virus (*Bunyaviridae* Family) Promoter Reveals Two Elements of Functional Importance

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We have performed an extensive mutational analysis of the proposed promoter region of the phlebovirus Uukuniemi (UUK), a member of the *Bunyaviridae* family. This was achieved by using a recently developed RNA polymerase I (Pol I)-driven reverse genetics system (R. Flick and R. F. Pettersson, J. Virol. 75:1643-1655, 2001). Chimeric cDNAs containing the coding region for the reporter chloramphenicol acetyltransferase (CAT) in an antisense orientation were flanked by the 5′- and 3′-terminal nontranslated regions of the UUK virus-sense RNA (vRNA) derived from the medium-sized (M) RNA segment. The chimeric cDNAs (Pol I expression cassettes) were cloned between the murine Pol I promoter and terminator, and the plasmids were transfected into BHK-21 cells. CAT activity was determined after cotransfection with viral expression plasmids encoding the RNA-dependent RNA polymerase (L) and the nucleoprotein (N) or, alternatively, after superinfection with UUK virus helper virus. Using oligonucleotide-directed mutagenesis, single point mutations (substitutions, deletions, and insertions) were introduced into the viral promoter region. Differences in CAT activities were interpreted to reflect the efficiency of mRNA transcription from the mutated promoter and the influence on RNA replication. Analysis of 109 mutants allowed us to define two important regulatory regions within the proximal promoter region (site A, positions 3 to 5 and 2 to 4; site B, positions 8 and 8, where underlined nucleotides refer to positions in the vRNA 3′ end). Complementary double nucleotide exchanges in the proximal promoter region, which maintained the possibility for base pairing between the 5′ and 3′ ends, demonstrated that nucleotides in the two described regions are essential for viral polymerase recognition in a base-specific manner. Thus, mere preservation of panhandle base pairing between the 5′ and 3′ ends is not sufficient for promoter activity. In conclusion, we have been able to demonstrate that both ends of the M RNA segment build up the promoter region and are involved in the specific recognition by the viral polymerase.

The *Bunyaviridae* family of arthropod- or rodent-borne viruses comprises more than 300 viruses (46) classified into five genera, *Bunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus*. *Bunyaviridae* members all share many common structural, molecular, and cell biological characteristics. They are enveloped viruses containing a tripartite, single-stranded RNA genome of negative polarity. The large (L) RNA segment encodes the RNA-dependent RNA polymerase (L), the medium-sized (M) segment encodes the two surface glycoproteins (G1 and G2) and in some member viruses also a nonstructural protein (NS_M), and the small (S) segment encodes the nucleoprotein (N) and in some cases a nonstructural protein (NS_S) (40). Upon entry of the virus into cells, the three virion ribonucleoprotein (RNP) species are released into the cytoplasm where the RNP-associated RNA polymerase catalyzes primary transcription, resulting in the synthesis of the individual mRNAs. Once new viral proteins have been translated, the virion RNA (vRNA) segments also serve as templates for the synthesis of full-length cRNAs, which in turn serve as templates for the synthesis of more vRNAs. This replicative cycle is also catalyzed by the viral RNA polymerase (40).

Uukuniemi (UUK) virus (a *Phlebovirus*), which is nonpathogenic for humans, has for more than three decades been used as an excellent model for studying the molecular and cell biology of the highly pathogenic members of the *Bunyaviridae* family. Initiation of transcription of the UUK virus-specific mRNAs is primed on vRNA templates by short sequences derived from the 5′ end of host mRNAs (41). This "cap snatching" occurs in the cytoplasm (36), and the endonucleolytic cleavage of the host mRNA some 10 to 20 bases downstream of the 5′ cap structure is probably carried out by the L protein, as described for other bunyaviruses (1, 16, 19, 20, 22, 45). As revealed by electron microscopy, the viral RNAs (18) and RNPs (35) are circular due to base pairing between complementary sequences at the 5′ and 3′ ends of each segment (6). These terminal sequences are conserved between each RNA segment and also between members within the same genus (5, 6). Thus, it is thought that this panhandle structure harbors the promoter elements necessary for initiation of both transcription and replication as well as genome packaging signals.

Bunyaviridae mRNAs derived from each vRNA segment are truncated at the 3′ end, due to as yet poorly characterized transcription termination signals (40). This means that the mRNAs are unable to circularize and thus to serve as templates for replication or transcription. Since they do not asso-

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TABLE 1. Oligonucleotide primers used to insert UUK promoter mutations and for vRNA and cRNA analyses

Primer	Sequence	Description
RF35	AATGAAGACGG⌊GGGACACAAAGACGGCTACCATGG BbsI 3′UUK NTR (vRNA)	Wild-type 3′ UUK NTR (vRNA)
RF107	AATGAAGACAT⌊AGGTACACAAAGACACGGCTACATGGAAC BbsI 5′UUK NTR (vRNA)	Wild-type 5′ UUK NTR (vRNA)
RF147	AATGAAGACGG⌊GGGAGACAAAGACGGCTACCATGGAGA BbsI 3′UUK NTR (vRNA)	Example of 3′ UUK mutation (G2C)
RF155	AATGAAGACAT⌊AGGTACACGAAGACACGGCTACATGGAAC BbsI 5′UUK NTR (vRNA)	Example of 5′ UUK mutation (A5G)
RF177	CATGGAAGCCATCACAGAC CAT ORF position 245 to 263 (UUK NTR M-CAT [vRNA])	PCR primer for amplifying UUK M-CAT vRNA molecules
RF267	ACGGCTACCATGGAGAAAAAAATC 3UUK NTR (vRNA) CAT ORF position 853 to 831 (UUK NTR M-CAT [vRNA])	Primer for RT of UUK M-CAT vRNA molecules
RF178	AAGACGGTGAGCTGGTGAT CAT ORF position 250 to 268 (UUK M-CAT [cRNA])	PCR primer for amplifying UUK M-CAT cRNA molecules
RF268	GACACGGCTACATGGAACAACAAATA 3′UUK NTR (cRNA) position 855 to 830 (UUK M-CAT [cRNA])	Primer for RT of UUK M-CAT cRNA molecules

ciate with the N protein and therefore are not packaged into virions, the encapsidation signal for N protein association is also likely to map to the 5′-3′ panhandle region, a conclusion that has recently also been confirmed experimentally (33, 42).

The lack of an efficient reverse genetics system for *Bunyaviridae* members has so far precluded the detailed mutational dissection of *cis*-acting elements in the promoter (panhandle) region of the three RNA genome segments. However, during the past few years the first reports have appeared describing attempts to develop such systems (4, 29, 37). By using the T7-vaccinia virus-based expression system (15), Bridgen and Elliott described a reverse genetics system for Bunyamwera (BUN) virus (*Bunyavirus*) that allowed the rescue of infectious BUN virus entirely from cloned cDNAs (2). We recently reported an alternative approach (11) based on the RNA polymerase I (Pol I)-driven expression system initially developed for influenza A virus (32, 48). This system allowed us to drive the replication and transcription of a reporter cDNA cassette (chloramphenicol acetyltransferase [CAT] or green fluorescent protein [GFP]) flanked by the 5′ and 3′ nontranslated sequences from the M RNA segment of UUK virus. The transcription of the reporter cassette, flanked by the murine Pol I promoter and terminator, is first carried out in the nucleus by RNA Pol I. This results in an uncapped, nonpolyadenylated, and nonspliced (Flick and Hobom, unpublished data) RNA transcript with the correct viral 5′- and 3′-terminal sequences (8, 48). After transport to the cytoplasm, the reporter RNA is replicated and transcribed by the L and N proteins supplied either by superinfection with UUK helper virus or by L- and N-expressing plasmids alone (11).

Here we have utilized the Pol I system to dissect the importance of the 5′ and 3′ nucleotide sequences of the M RNA segment of UUK virus for replication and transcription. A total of 109 point mutations, deletions, or insertions were introduced in the putative promoter region and their effects on reporter (CAT) expression were determined. The result of these extensive mutational analyses identified two crucial sites in the panhandle region separated by a short spacer region, the length of which is critical.

MATERIALS AND METHODS

Cells and virus. BHK-21 cells (American Type Culture Collection) were grown on plastic dishes in Eagle's minimal essential medium (EMEM) supplemented with 7.5% fetal calf serum (Invitrogen/Life Technologies), 2 mM L-glutamine, 100 IU of penicillin/ml, and 100 μg of streptomycin/ml. The origin and the preparation of stock virus from the prototype strain S23 of UUK virus have been described (34). The stock virus had a titer of 2×10^8 PFU/ml. Cells were infected with a multiplicity of infection of approximately 5 PFU/cell.

Construction of plasmids. Mutations in the 5′ and 3′ vRNA promoter regions were generated by using pRF108 as a basic vector construct. This plasmid contains the ribosomal DNA (rDNA) promoter region (−251 to −1 relative to the 45S pre-rRNA start point) and the rDNA terminator sequence (+571 to +745 relative to the 3′ end of the 28S rDNA) derived from murine rDNA (48). Between these two elements is a spacer region flanked by *Bsm*BI and *Bbs*I sites. After restriction by *Bsm*BI and *Bbs*I, *Bbs*I- or *Bsm*BI-restricted PCR fragments can be inserted in an orientation-specific manner. Using primers carrying the promoter single point mutations, the template pRF200 (Pol I [murine] UUK M-CAT, recently described as pRF33;11) can be PCR amplified, introducing the mutations into the nontranslated chimeric M-segment ends. Double substitution mutants were constructed by ligation of the large *Bgl*II/*Eco*RI fragment (3,200 bp) from the 3′ single mutation constructs and the small *Bgl*II/*Eco*RI fragment (657 bp) from the 5′ single mutation plasmids.

After transfection into BHK-21 cells, the resulting constructs can be transcribed by the cellular RNA Pol I, generating RNA transcripts without any additional nucleotides or modifications at the 5′ or 3′ ends [e.g., cap structure, poly(A) tail]. Consequently, artificial UUK vRNA segments with exchanged open reading frames (ORFs) (glycoprotein precursor replaced with CAT) flanked by promoter mutations are produced.

Whenever PCR was used the sequence of the inserts was checked by dideoxy sequencing with an ABI PRISM3100 sequencer (Applied Biosystems). In cases where a central part of the inserted PCR fragment was exchanged with a nonamplified fragment derived from previously characterized inserts, only the sequences of the flanking regions were checked. The oligonucleotide primers used in the paper are listed in Table 1.

Transfection. Subconfluent (60 to 80%) BHK-21 cells (3×10^6) were cotransfected with plasmids (1 μg) containing promoter mutations and viral expression plasmids pCMV UUK-L and pCMV UUK-N (2.4 and 0.3 μg, respectively) (11) by using 20 μl of liposome Plus buffer (Lipofectamine PLUS; Invitrogen/Life Technologies) mixed in serum-free EMEM. After 15 min, 12 μl of liposome reagent was added and incubation was continued for another 15 min. The

BHK-21 cells were incubated at 37°C with the DNA-Lipofectamine mixture for 3 to 5 h. After further incubation for 20 h in EMEM containing 7.5% fetal calf serum, the transfected cells were washed with phosphate-buffered saline and harvested for CAT analysis or used for UUK virus superinfection. To determine the efficiency of transfection, the plasmid pHL2823, which contains an enhanced GFP under the cytomegalovirus (CMV) promoter (Flick and Hobom, unpublished data), was similarly transfected.

Superinfection with UUK helper virus. Plasmids containing promoter mutations were transfected into subconfluent BHK-21 cells (6×10^6) by using the technique described above, with the following modified amounts of reagents: 4 μg of the respective plasmid, 20 μl of the liposome Plus buffer (Lipofectamine PLUS; Invitrogen/Life Technologies), and 30 μl of liposome reagent. Twenty hours posttransfection the cells were washed with adsorption medium (EMEM supplemented with 20 mM HEPES and 0.2% bovine serum albumin) and superinfected with UUK virus at a multiplicity of infection of 5 PFU/cell. After a 60-min adsorption period, the cells were washed once and incubated with fresh adsorption medium for 15 to 30 h. A complete replication cycle occurs during this period.

CAT assay. Cell extracts were prepared as described by Gorman et al. (17). In a first experiment, 50% of each cell lysate (prepared from 3×10^6 cells in the case of cotransfection experiments and 6×10^6 cells in superinfection experiments) and, depending on the results, serially diluted samples of the various cell lysates were mixed with 10 μl of acetyl-coenzyme A (4 mM lithium salt; Sigma) and 10 μl of fluorescently labeled chloramphenicol substrate (borondipyrromethane difluoride fluorophore [BODIPY CAM substrate]; Flash CAT kit; Stratagene) and then incubated at 37°C for 2 h. For extraction of reaction products, 0.4 ml of ethyl acetate (Merck) was added, and after centrifugation for 1 min at 15,000 × *g*, the upper phase containing the reaction products was isolated and the solvent was evaporated. The resulting pellet was resuspended in 20 μl of ethyl acetate, and the reaction products were separated by thin-layer chromatography (20- by 20-cm plates; Silica gel 60; Merck) using a solvent mixture of chloroform and methanol (87:13). Finally, the reaction products were visualized by UV illumination, documented by photography, and evaluated using Quantity One software (Bio-Rad). Ratios of CAT activities were calculated based on at least three independent sets of serial dilutions of cell lysates down to a level of 30 to 50% product formation. The percentages of CAT activity in the figures reflect the ratios between product and substrate relative to the positive control pRF200 (wild-type promoter construct = 100%).

RT-PCR and semiquantitative PCR analysis. BHK-21 cells were transfected with promoter mutant-containing Pol I cassettes and viral expression plasmids for UUK-L and UUK-N (pCMV UUK-L, pCMV UUK-N) as described before (11). Total RNA was isolated (RNeasy mini kit; Qiagen) at 20 h posttransfection and treated with DNase (Amp grade; Gibco BRL). RNA was quantitated by UV absorbance (A_{260}/A_{280}) using an Eppendorf Biophotometer, and 1 μg of total RNA was used as template for the reverse transcription (RT) (rTth kit; Applied Biosystems). Artificial UUK M-CAT cRNA and vRNA molecules were reverse transcribed (30 min, 60°C) using oligonucleotides RF268 and RF267, respectively. Oligonucleotides RF178 or RF177, respectively, were added under the following PCR conditions: 1 min at 94°C; 40 cycles of 30 s at 94°C and 30 s at 60°C; and 75 min at 60°C. The reaction products (for cRNA amplification, a 605-bp fragment; for vRNA amplification, a 608-bp fragment) were analyzed in a 1.5% agarose (Gibco BRL) horizontal gel containing 0.25 μg of ethidium bromide (Gibco BRL) per ml in Tris-borate-EDTA electrophoresis buffer, documented by digital photography, and analyzed using Quantity One software (Bio-Rad).

RESULTS

General strategy for expression of CAT cDNA flanked by UUK M vRNA nontranslated regions (NTRs). The reporter plasmid pRF200 (CAT-M vRNA) and all the 109 promoter mutant constructs described below contained the ORF of the reporter CAT gene in antisense orientation, flanked by the 5′- and 3′-terminal sequences of the UUK virus M vRNA segment. Oligonucleotide-directed mutagenesis of the terminal sequences was carried out by introducing mutations into PCR primers. The chimeric constructs were expressed by using the murine Pol I system (11, 32, 48). Plasmids were introduced into BHK-21 cells by liposome-mediated transfection. Control experiments using a CMV-enhanced GFP expression plasmid (pHL2823) (Flick and Hobom, unpublished data) indicated a reproducible transfection efficiency of approximately 20 to 25% (11). To drive transcription and replication of the chimeric RNAs, cells were either infected with UUK virus 20 to 24 h after transfection or cotransfected with expression plasmids encoding viral L and N proteins (11). The observed CAT activity directly reflects the CAT enzyme concentration, which is dependent on the viral mRNA transcription rate. The latter is in turn dependent on the vRNA promoter activity. Therefore, the CAT activity reflects the interaction between the viral RNA polymerase and the promoter region and this was used to quantify the effect of the introduced promoter mutations on the viral promoter activity. To make it easier for the reader to assess the effect of the mutations on promoter activity, we have used a color code to describe the CAT activity obtained with each mutant. Each mutant was given a number, which appears in the figures showing the quantification of CAT activity (Fig. 1 to 3) and in the summary figures (Fig. 1E and 4) (see figure legends for further details).

Mutational analysis of the proximal promoter element—identification of two important promoter elements, A and B. In the first series of mutants, single point mutations were introduced into the proximal promoter region of the UUK virus M segment. The 5′ and 3′ sequences of this region, which encompass positions 1 to 10, are completely complementary (see Fig. 1E, 4, and 6) and are likely to be crucial in the formation of the panhandle structure and the circularization of the vRNA and cRNA species. Analyses of CAT activity in lysates prepared from cells expressing these mutants revealed nucleotide positions which could be mutated with no or only a moderate effect on the promoter activity (e.g., positions 1, 2, 6, 7, 9, and 10 and 1, 5 to 7, 9, and 10, where underlined nucleotides refer to positions in the vRNA 3′ end) and other positions which were very sensitive to any kind of modification (positions 3 to 5 and 8 and 2 to 4 and 8) (Fig. 1A to D; summarized in Fig. 1E). Interestingly, two distinct elements within the proximal promoter part in the panhandle conformation could be identified as important regulatory sites (site A, positions 3 to 5 and 2 to 4; site B, positions 8 and 8) (Fig. 4). These elements are separated by a short stretch of nucleotides that are apparently not recognized by the viral polymerase in a nucleotide-specific manner (positions 6 to 7 and 5 to 7).

To determine if these nucleotides only serve a spacer function to position the important promoter elements A and B to the correct interaction sites on the viral polymerase, additional nucleotides were inserted between positions 5 and 6 into the 5′ or 3′ promoter arms and the new constructs were tested for CAT expression. As a result, a substantial difference between the insertions into the 5′ versus the 3′ part of the UUK vRNA promoter could be demonstrated (Fig. 1E and 2A). Nucleotide insertions between positions 5 and 6 at the 5′ end completely abolished the promoter activity, since no CAT activity could be detected, independent of whether an insertion of one or two A (pRF251, pRF252) or U (pRF253, pRF254) residues was introduced. In contrast, the insertion at the corresponding position at the 3′ end of the vRNA resulted in strong remaining promoter activity, independent of whether one or two A (pRF259, pRF260) or U (pRF257, pRF258) residues were inserted. Only insertion of three or five nucleotides between positions 5 and 6 dramatically decreased reporter expression in

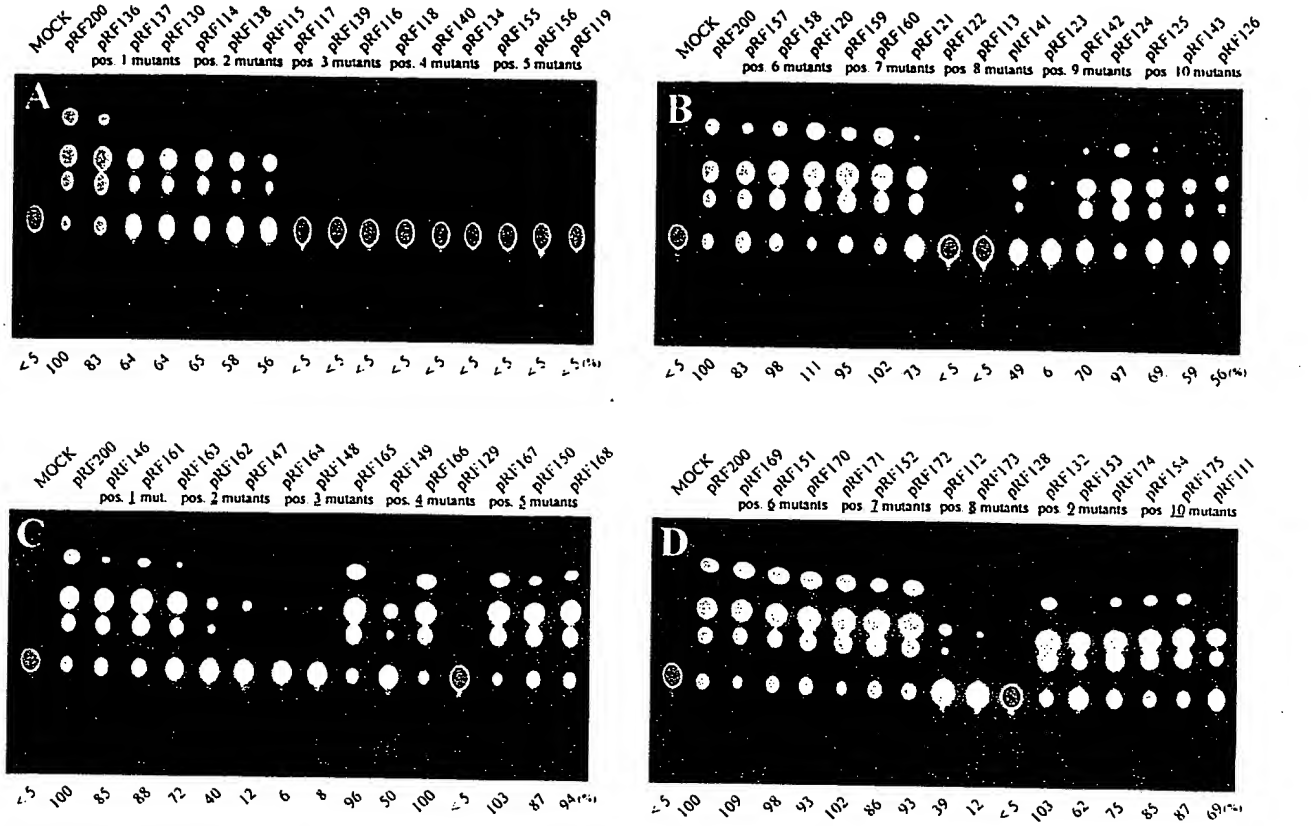

FIG. 1. Comparative CAT analysis of single nucleotide substitution derivatives of UUK M-CAT minigenomes in the proximal promoter region and summary of the effect on CAT expression of point mutations introduced into the promoter region of the UUK M-segment-based minigenome. BHK-21 cells were transfected with different RNA Pol I-driven mutated promoter constructs containing UUK M-CAT minigenome plasmids and cotransfected with viral expression plasmids pCMV UUK-L and pCMV UUK-N. At 30 h posttransfection, the cells were harvested and analyzed for CAT activity. Acetylated products were separated by thin-layer chromatography. Construct numbers are indicated above each lane. CAT activity, expressed as the percentage relative to the wild-type promoter construct pRF200, is indicated below each lane. (A to D) CAT activity in BHK-21 cells transfected with pCMV UUK-L and pCMV UUK-N expression plasmids and UUK M-CAT minigenomes containing mutations in the 5′ branch of the proximal promoter element (positions 1 to 5 at the 5′ vRNA end) (A), mutations in the 5′ branch of the proximal promoter element (positions 6 to 10 at the 5′ vRNA end) (B), mutations in the 3′ branch of the proximal promoter element (positions 1 to 5 at the 3′ vRNA end) (C), and mutations in the 3′ branch of the proximal promoter element (positions 6 to 10 at the 3′ vRNA end) (D). Colors: red, no detectable CAT activity (<5%); orange, CAT activity between 5 and 49%; yellow, CAT activity between 50 and 80%; green, CAT activity above 80%. The CAT activity of the basic construct pRF200 (UUK M-CAT with wild-type promoter sequence) was set at 100%. Mock, BHK-21 cells transfected only with pRF200. (E) Summary of the effect on CAT expression of point mutations introduced into the promoter region of the UUK M-segment-based minigenome. The nucleotide sequence of the base-paired panhandle configuration formed by the inverted complementary 5′ and 3′ ends of the UUK M vRNA is shown in the middle (in black and white). The compiled CAT expression efficiencies (average of three or more experiments for each mutant) have been tabulated above and below that sequence for a complete set of 77 single-substitution mutants in the 5′ and 3′ arms, respectively. In addition, the results from 15 double-substitution mutants, 14 insertion mutants (hexagons), and 3 deletion mutants (triangles) in the proximal and distal promoter elements as well in the bulge region are listed. CAT activities are relative to the pRF200 reporter gene expression rate (wild-type promoter) as shown in the color legend. For representative experimental data, see panels A to D and Fig. 2. Note that due to the mechanism of RNA Pol I transcription termination, the substitution U1C cannot be analyzed (48).

the case of A insertions (pRF266, pRF267) or completely abolished promoter activity in the case of U insertions (pRF268 and pRF269) (Fig. 1E and 2A).

The lack of correct interaction between the viral polymerase and the substituted nucleotides could be the result of changing the nucleotide sequence or changing base-pairing potential in the panhandle. Mutants with complementary double substitutions with preserved base pairing were therefore generated to examine the promoter-abolishing effect of the previous introduced single point mutations. As shown in Fig. 2B and summarized in Fig. 1E, complementary double substitution constructs containing an inactivating single point mutation (positions 3 to 5 and 8) resulted in undetectable CAT activity (pRF180, pRF220, pRF178, and pRF177). Thus, the nature of the nucleotide located at a certain promoter position is more important than base pairing per se. All other constructs with altered base pairs showed CAT activity comparable to that obtained for the single point mutations (positions 1, 7, and 9) or had slightly decreased CAT expression levels (positions 2, 6, and 10).

In summary, the mutational analyses of the highly conserved proximal promoter region of the UUK virus M vRNA segment demonstrate that two important polymerase interaction sites (site A and site B) are located within this region. Sites A and

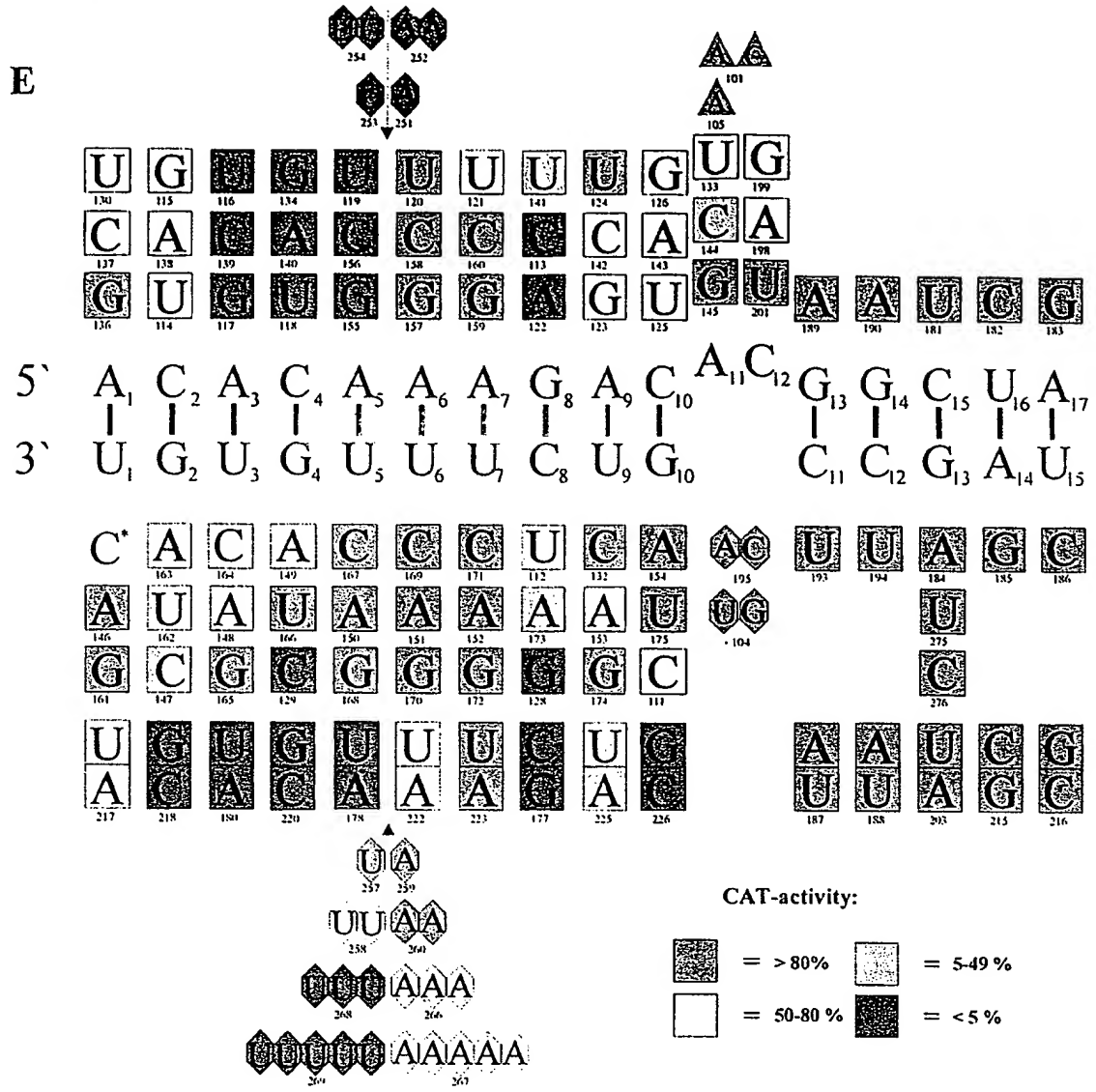

FIG. 1—*Continued.*

B are separated by a stretch of nucleotides which are not recognized by the viral polymerase in a nucleotide-specific manner. The distance between sites A and B seems to be important only for the 5′ part of the promoter, whereas the 3′ part is less sensitive to nucleotide insertions, suggesting a binding mechanism differing between vRNA and cRNA molecules.

Mutational analysis of the bulge region. Nucleotides A11 and C12 at the 5′ end are not complementary to bases at the 3′ end of the UUK M vRNA, while the next 5 nucleotides (which we call the distal promoter region) are complementary. Thus, these two nucleotides are likely to form a bulge (Fig. 1E and 4). This bulge constitutes the main difference between the vRNA and the cRNA promoter, whereas residues 1 to 10 (proximal promoter region) and the distal promoter region at the 5′ and 3′ ends of the RNA in both promoter situations consist of fully complementary nucleotides (Fig. 1E and 4). Therefore, it was expected that these bulge structures should play an important role for the interaction with the viral RNA polymerase, perhaps to discriminate between different RNA species during the packaging process as shown for influenza A virus (43). Using an oligonucleotide-directed mutagenesis approach, single point mutations, including substitutions, deletions, and insertions, were introduced into this bulge region. Interestingly, substitutions maintaining a purine at position 11 (A-to-G substitution; pRF145) or a pyrimidine at position 12 (C-to-U substitution; pRF201) had only minor effects on promoter activity, whereas constructs with substitutions of a pyrimidine at position 11 (pRF133, A11U; pRF144, A11C) or of a purine at position 12 (pRF198, C12A; pRF199, C12G) gave a slight to moderate decrease in CAT activity compared to the wild-type promoter construct pRF200 (Fig. 1E and 2C).

The role of the bulge region was also examined by generating constructs with insertions and deletions. Surprisingly, deletion of the A11 nucleotide (pRF105) as well as the deletion

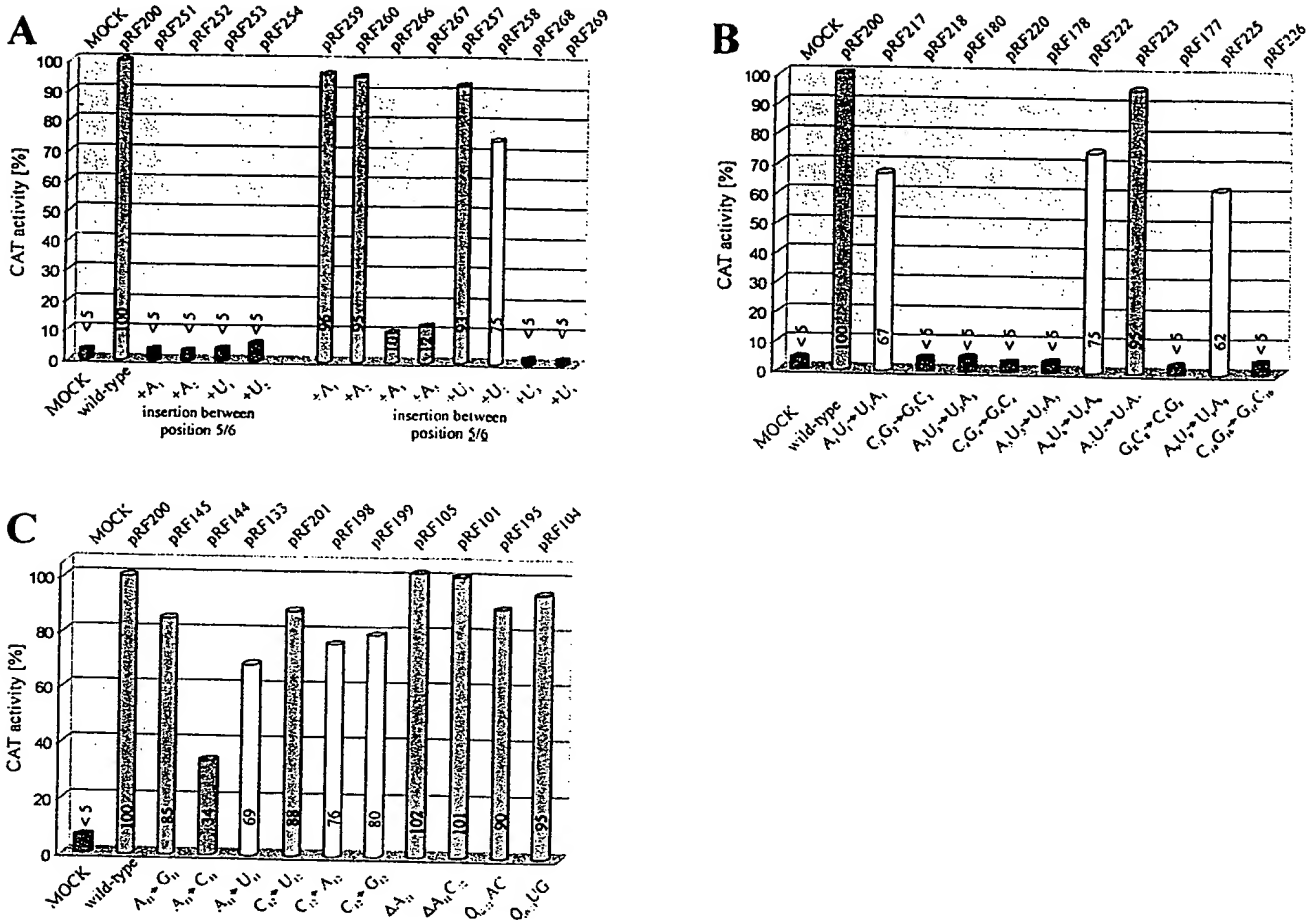

FIG. 2. Graphic representation of CAT activities from UUK minigenomes containing nucleotide mutations in the 5′ and/or 3′ arms of the UUK M-CAT minigenome promoter region. (A) RNA Pol I-driven UUK M-CAT minigenome plasmids containing nucleotide insertions in the proximal promoter element were transfected into BHK-21 cells and cotransfected with pCMV UUK-L and pCMV UUK-N expression plasmids. At 30 h posttransfection cells were harvested and analyzed for CAT activity. The analyzed constructs are listed on the *x* axis. Construct numbers are shown on top of the graph, and the number of inserted nucleotides and type of nucleotide are listed below the graph. The *y* axis shows the CAT activities from each construct compared to that of the wild-type minigenome pRF200. (B) Graphic representation of CAT activities from UUK minigenomes containing compensating double mutations in the proximal promoter element. (C) Analysis of the effect of mutations introduced into the bulge region of the viral M-segment promoter.

of both bulge nucleotides A11 and C12 (pRF101) had no effect on the promoter activity (Fig. 1E and 2C). Furthermore, nucleotide insertions into the 3′ part of the vRNA promoter opposite to the two bulge nucleotides (between positions 10 and 11) showed minor effects on promoter activity. This was true both in the case of nucleotide insertion with base-pairing potential of the bulge nucleotides A11 and C12 (pRF104) and in the case of insertion without any base-pairing possibility (pRF195) (Fig. 1E and 2C). With the constructs pRF101 (ΔA11C12) and pRF104 (insertion of UG at positions 10 and 11), a UUK promoter sequence was generated that displayed no differences between the vRNA and cRNA promoter situation. In these mutants, either the bulge nucleotides were deleted or nucleotides complementary to the bulge region were inserted, thus generating the possibility for a completely double-stranded promoter region of 15 or 17 bp, respectively.

In conclusion, the results from the single nucleotide exchange experiments suggest that the bulge region is involved in the interaction between the viral polymerase and the promoter region. However, there is no nucleotide-specific recognition by the viral polymerase. Instead, the type of nucleotide (purine or pyrimidine) appears to be important. In contrast, the deletion and insertion experiments demonstrated that the unpaired bulge nucleotides are not necessary for the transcription and replication processes of the UUK minigenomes (Fig. 1E, 2C, and 4). However, their role in the packaging process has to be further analyzed.

Analysis of the distal promoter element. Similar to the mutational analysis of the proximal promoter region, the effect of single point mutations in the distal promoter element (positions 13 to 17 and 11 to 15) was also analyzed. This was followed by an analysis of complementary double substitutions, all of which preserved base pairing. The single nucleotide exchanges were chosen so that the interactions between the nucleotides from the 5′ promoter arm and the 3′ arm within the distal promoter element were either weakened, e.g.,

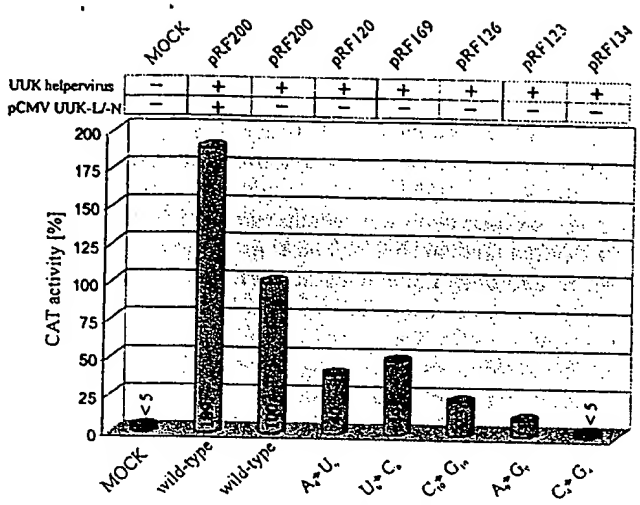

FIG. 3. CAT analysis of UUK minigenomes containing representative single point mutations in the proximal promoter element driven by UUK superinfection. BHK-21 cells were transfected with selected minigenomes containing mutated promoter sequences. At 24 h posttransfection cells were superinfected with UUK helper virus and then were harvested at 30 h postinfection and analyzed for CAT expression. CAT activity measured from each lysate is expressed as the percentage of the activity obtained from cells transfected with the wild-type promoter construct pRF200 and superinfected with helper virus (set at 100%) (lane 3).

G—C→G-U, or destroyed, e.g., G—C→A/C, where "—" indicates a strong bond, "-" indicates a weak bond, and "/" indicates the absence of a bond. It should be noted that in the latter case the cRNA promoter will still carry a week G-U basepair. Furthermore, since the authentic AUG start codon is located at positions 18 to 20 (5′ end of cRNA), single point substitutions were chosen such that the optimal Kozak sequence (23) was not influenced, i.e., keeping a purine at cRNA position 15 (Kozak position −3) and a pyrimidine at cRNA position 14 (Kozak position −4).

Surprisingly, all of the analyzed single point mutation constructs showed a promoter activity similar to that of the wild-type promoter construct pRF200 (not shown, but summarized in Fig. 1E and 4). This suggests that none of the nucleotides in the distal promoter region are recognized by the viral polymerase in a nucleotide-specific manner, nor does the base pairing between the 5′ and 3′ promoter arms in this region seem to play an important role (the A/C situation in the vRNA promoter [pRF189, pRF190, pRF184, pRF182 and pRF186]). CAT expression (not shown, but summarized in Fig. 1E) of complementary double substitution mutants with preserved base pairs between the 5′ and 3′ promoter arms in the distal promoter region was likewise comparable to that of the wild-type promoter (pRF187, pRF188, pRF203, pRF215, and pRF216), demonstrating again that the distal region is not recognized by the viral polymerase in a nucleotide-specific manner.

Mutational analysis combined with UUK superinfection. To demonstrate that the CAT activities determined for the different promoter mutants are not specific to the use of cotransfected plasmids expressing the UUK nucleoprotein (N) and the viral polymerase (L), we repeated the analysis with a set of selected mutants using UUK superinfection as a source for the viral N and L proteins. The mutant constructs selected were pRF120 and pRF169 (wild-type level of CAT activity), pRF126 (moderate reduction of CAT activity), pRF123 (strongly reduced CAT activity), and pRF134 (no detectable CAT expression). As expected, comparable relative CAT activities were observed (Fig. 3), but at a lower level than in the cotransfecting experiments, in agreement with our recently published data (11).

RNA analysis of promoter-inactivating mutations. The introduced promoter mutations can have effects at different stages of the viral life cycle. They can, for example, influence

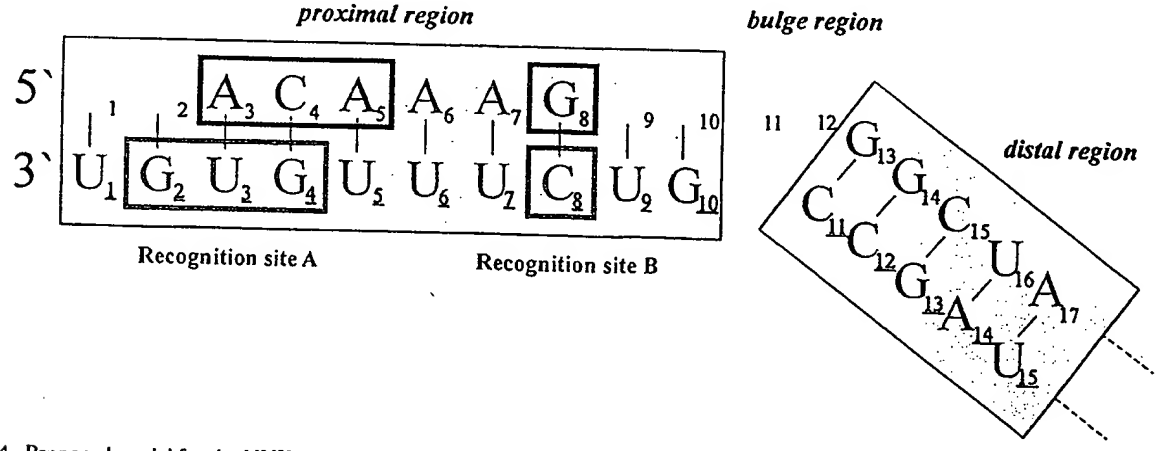

FIG. 4. Proposed model for the UUK promoter elements important for the interaction with the viral RNA polymerase as suggested by the CAT activity pattern of single substitutions and supported by complementary double substitutions and nucleotide insertions or deletions. The nucleotide sequence of the panhandle proposed to be formed between the 5′ and 3′ ends of the UUK M vRNA is shown. The proximal and distal promoter regions are shown in gray boxes, separated by the two bulge nucleotides. Promoter elements framed by red and orange boxes represent the nucleotides most important for promoter activity. They are referred to as recognition sites A and B. Green and yellow letters show freely exchangeable promoter nucleotides that, when mutated, have no or little effect on promoter activity.

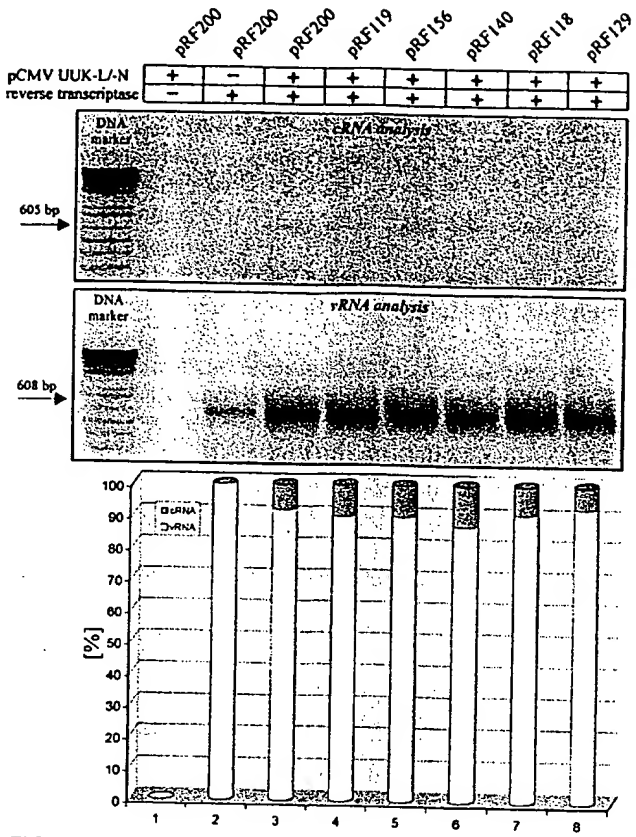

FIG. 5. Analysis of vRNA and cRNA synthesis by CAT-negative promoter mutants. BHK-21 cells were transfected with selected CAT-negative promoter mutant constructs and cotransfected with viral expression plasmids as shown at the top. Total RNA was isolated at 20 h posttransfection and treated with DNase I to destroy the transfected plasmids. Subsequently, 1 μg of total RNA was used as a template for RT, followed by a PCR with UUK-CAT minigenome-specific oligonucleotides (see Table 1). The reaction products (cRNA amplification, 605-bp fragment; vRNA amplification, 608-bp fragment) were analyzed in a 1.5% agarose horizontal gel. The upper panel shows the cRNA amplification product, the middle panel shows the vRNA amplification product, and the lower panel shows the vRNA/cRNA ratio of all examined promoter mutant minigenomes.

the overall binding of the viral polymerase to the promoter region, or they can affect the specific interaction with the active center of the polymerase. Different polymerase reactions can also be influenced by these mutations, e.g., transcription, replication, or cap-snatching or endonucleolytic cleavage. In addition, the packaging process may be affected by the altered promoter sequence. Furthermore, nucleotide substitutions can have different effects on the vRNA and cRNA promoters. Therefore, we examined vRNA and cRNA levels in BHK-21 cells transfected with promoter mutants showing no detectable CAT activity. We used a semiquantitative RT-PCR approach to examine the vRNA/cRNA ratios for each analyzed single promoter-inactivating mutant. For the RT reaction, the primer RF267 was used to start the RT reaction at the 3′ end of the vRNA of the M-CAT segment and primer RF268 was used for the RT of minigenome cRNA. For the following PCR amplification step, RF177 (for vRNA detection) or RF178 (for cRNA detection) was used as the reverse primer to amplify a 608- or 605-bp fragment, respectively. Since the 3′ end of the M mRNA has been found to be about 100 nucleotides shorter than the full-length cRNA (R. Rönnholm and R. F. Pettersson, unpublished data), the primer RF268 was designed such that no viral mRNA could be amplified. This ensured that the amplification product represented only the cRNA derived from the artificial UUK M-CAT segment.

Control experiments were conducted to exclude any plasmid DNA contamination (Fig. 5, lane 1) or any cRNA background (Fig. 5, lane 2) by not adding the reverse transcriptase to the RT-PCR or by using total RNA from BHK-21 cells transfected with only the wild-type promoter construct pRF200 (no cotransfected expression plasmids), respectively. As expected, no amplification product could be detected after an RT-PCR control reaction in the absence of reverse transcriptase, showing that no DNA contamination was present (Fig. 5, lane 1). Furthermore, the second control experiment analyzing RNA from cells transfected only with pRF200 (wild-type promoter), omitting the two expression plasmids, demonstrated that only vRNA molecules could be amplified due to the expressed RNA Pol I transcripts, whereas no cRNA could be detected (Fig. 5, lane 2). The different levels of vRNA with and without cotransfected viral N and L expression plasmids (lane 2 versus lane 3) demonstrate clearly the replication activity (vRNA→cRNA→vRNA) in our minigenome rescue system.

The selected mutant constructs that had promoter-abolishing effects were pRF140 (C4A), pRF118 (C4U), pRF119 (A5U), pRF156 (A5C), and pRF129 (G4C) (Fig. 1 and 4). As expected, the vRNA amplification product was easily detectable for all examined promoter mutants due to the RNA transcribed by RNA polymerase I. However, the amount of cRNA was much lower than that of the vRNA, as expected for a negative-strand RNA virus (30) (Fig. 5, upper panel). To determine the effect of the introduced promoter mutations on the viral replication steps (vRNA→cRNA, cRNA→vRNA), the vRNA/cRNA ratio was determined for the five mutants and compared to that derived from the wild-type promoter construct pRF200. In our system, a vRNA/cRNA ratio of about 90:10 was obtained for pRF200 (wild-type promoter) in cells cotransfected with the pCMV UUK-L and pCMV UUK-N expression plasmids (Fig. 5, lower panel, column 3). Surprisingly, similar levels of cRNA synthesis were detected for all five examined promoter mutants compared to the wild-type situation, demonstrating that the first replication step (vRNA→cRNA) is not severely blocked by the introduced mutations (Fig. 5, columns 4 to 8). Furthermore, the vRNA/cRNA ratios, which reflect the effect on both replication steps, were in a close range between 90:10 and 85:15 for all examined promoter mutants, demonstrating no major influence of the nucleotide exchanges on the minigenome replication.

Thus, we conclude that the reason for the lack of detectable CAT activity of some promoter mutants is not the result of blocked replication but is rather likely to be due to the inability to initiate and synthesize functional viral mRNA.

DISCUSSION

The development of a reverse genetics system for UUK virus (11) based on the RNA pol I system (32, 48) has opened up the possibility to genetically manipulate this and other members of

the *Bunyaviridae* family. As a first application of this new system, we have introduced a total of 109 mutations into the putative promoter region of the M segment and studied their effects on the promoter function as determined by analyzing the expression of a minigenome encoding CAT. In these analyses, we have focused on the 5′ and 3′ ends of the vRNA molecule, which we call the 5′ and 3′ promoter arms, respectively. The two ends are able to form a stable panhandle structure due to base pairing between inverted complementary nucleotide sequences (5, 6, 18, 39). The two promoter arms are thought to regulate replication and transcription as well as the association of the vRNA with the N protein to form RNPs. Furthermore, they probably contain the putative RNA segment packaging signal.

The results of our mutational analyses can be summarized as follows (Fig. 4). Two critical sites, or elements, called A and B, were identified in the proximal region of the promoter, which comprises the 10 terminal residues of the 5′ and 3′ ends of the vRNA. At the 5′ promoter arm, nucleotides at positions 3 to 5 (site A) and 8 (site B) could not be changed to any other residue without abolishing or substantially reducing promoter activity. Likewise, mutating residues 2 to 4 (site A) and 8 (site B) in the 3′ promoter arm drastically reduced activity, although some changes at positions 3 and 4 were tolerated. The abolishing effect of double mutations in the 5′ and 3′ arms, which preserved base pairing, also underscored the importance of the nucleotide sequence at the two sites. The spacing between sites A and B (two nucleotides) in the 5′ promoter arm was found to be critical, as introduction of just a single base abolished promoter activity. In contrast, the corresponding spacer region (three residues) in the 3′ promoter arm tolerated the introduction of one or two residues without significant loss of activity. Taken together, these results support the notion that the sequence of the 5′ promoter arm is more critical than that of the 3′ promoter arm (see below). A surprising finding was that the bulge consisting of two unpaired residues at positions 11 and 12 (5′ promoter arm) could be deleted, base paired, or mutated without loss of activity. However, a purine at position 11 and a pyrimidine at position 12 were still favored in single point mutation experiments. Finally, each nucleotide of the distal promoter region which has the potential to fully base pair could be mutated without loss of activity, even if base pairs were destroyed. No experimental proof could be found that both RNA segment ends have to be in a panhandle conformation to serve as a functional promoter. However, important residues for the promoter function, which could function independently during the interaction between the viral polymerase and the vRNA or cRNA promoter, are located in each promoter arm.

Our analysis of the UUK M vRNA promoter function is the first extensive in vivo mutagenesis study of a *Bunyaviridae* member based on the exchange of all nucleotides within the entire promoter region. One previous study on Rift Valley fever (RVF) virus focused on the effect of deletions within the promoter region (37). Here, recombinant vaccinia viruses providing the RVF virus L and N proteins and in vitro synthesized T7 runoff transcripts (minigenomes) were used and only transcription rather than replication and packaging of the minigenomes could be analyzed. The results indicated that the promoter region between nucleotides 3 and 8, as well as the G residue at position 13 in the 3′ promoter arm of the genome-sense RNA (vRNA), was important for transcription initiation. The former region corresponds to our sites A and B, including the spacer (residues 2 to 8), and the results thus support the conclusion of the importance of this region in phlebovirus RNA segments.

Extensive mutagenesis studies similar to the one reported here have during the last decade been carried out with influenza A virus (9, 10, 14, 21, 25–28, 31, 38, 44). These analyses either employed the RNA Pol I-driven reverse genetics system used here (32, 48) or a T7-driven system (15). Because of the similarities between the orthomyxoviruses and *Bunyaviridae* members, it is of interest to compare the results obtained with these two virus systems. The first conclusion is that the results clearly reveal many common features. Nucleotides important for promoter activity are in both cases mainly located at the 5′ promoter arm. In the case of UUK virus, there were 11 mutations of nucleotide positions in the 5′ promoter sites A and B that completely abolished activity, while only two mutations at the 3′ promoter arm led to no detectable reporter gene activity. For influenza virus, the corresponding numbers were three mutations in the 5′ arm and only one in the 3′ arm (10). Furthermore, two sites sensitive to single point mutations could be defined for the UUK M vRNA promoter, namely site A (positions 3 to 5 [5′ arm] and 2 to 4 [3′ arm]) and site B (position 8 [both 5′ and 3′ arms]) (Fig. 1E and 4). Similarly, two critical sites were identified in the influenza A virus promoter: site A (positions 2, 3, and 5 and 2 and 3) and site B (positions 7 to 9 and 7 to 9). For both viruses, these important sites for promoter activity are separated by a stretch of two to three nucleotides, the sequence of which is not critical for the recognition by the viral polymerase (Fig. 1E) (7). In the case of UUK virus, nucleotide insertions between sites A and B demonstrated that the distance between these sites plays an important role for the 5′ promoter arm (Fig. 1E and 2B) but much less so for the 3′ promoter arm. The fact that this promoter part is very sensitive to nucleotide changes suggests that the viral RNA polymerase interacts very specifically with the 5′ promoter arm.

Besides positions recognized in a nucleotide-specific manner and positions having just a spacer function, the promoter region contains nucleotides that can only be replaced by a similar type of nucleotide (e.g., positions 1, 2, and 11 must be purines and position 12 must be a pyrimidine). Again, similarities can be found by comparison with influenza A virus (9, 10).

Therefore, viral polymerases differentiate between the following three different types of promoter nucleotides: (i) nucleotides that interact in a sensitive and nucleotide-specific manner, (ii) nucleotides for which being a purine or pyrimidine is important, and (iii) a third type of nucleotides which are not directly recognized by the viral polymerase but serve a spacer function to position the functionally important promoter residues for the proper interactions.

The proximal promoter region (the first 10 nucleotides; Fig. 4) of the L, M, and S vRNAs of UUK virus is highly conserved except at position 9 in the 5′ promoter arm. At this position, the L segment has a U, whereas the M and S segments have an A. This natural variation can be understood based on the result of the different CAT expression levels of the single point mutations at position 9. As expected, the two naturally occurring

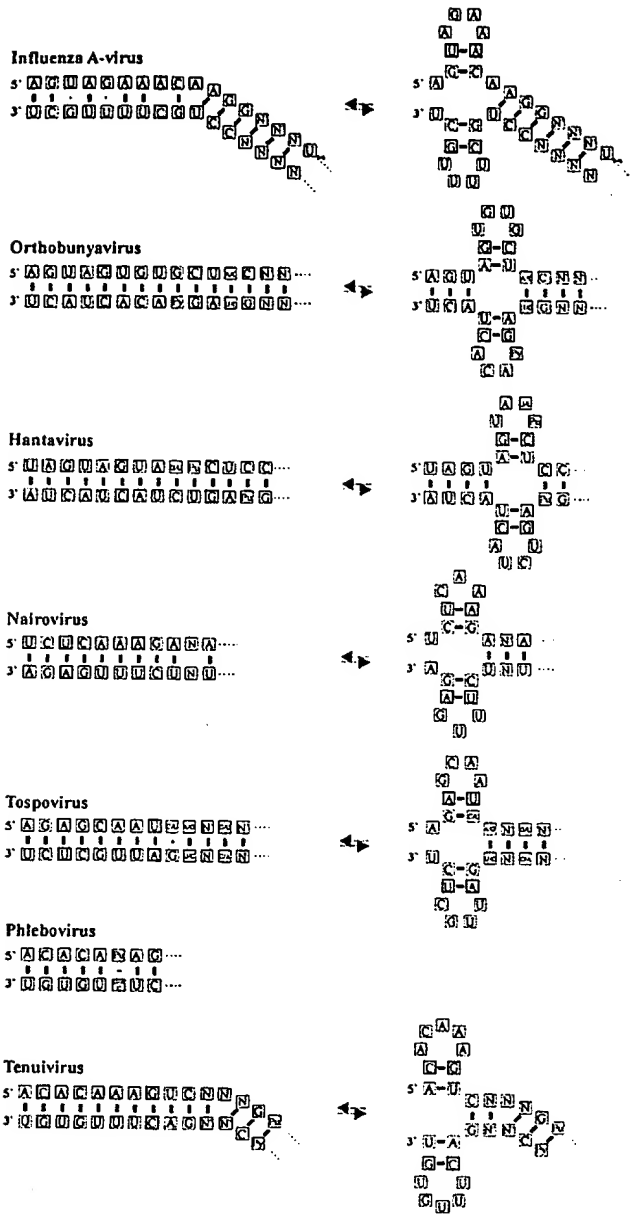

FIG. 6. Secondary structure predictions of the highly conserved terminal regions of the genome segments of members of the five *Bunyaviridae* genera and the Tenuiviruses in comparison to the corkscrew structure of the influenza A virus vRNA segment promoter. In four genera of the *Bunyaviridae* family, the terminal nucleotides of the RNA genome segments (vRNA and cRNA) can theoretically form intrastrand stem-loop structures, referred to as the corkscrew configuration. The model is based on the experimentally proven promoter structure of the influenza A (9, 10, 25, 26) and C (3) viruses and the thogoto (24, 47) virus.

nucleotides showed a similarly high CAT activity (100 and 97%; Fig. 1B and E), whereas an A9C or an A9G substitution resulted in decreased CAT activity (70 and 6%; Fig. 1B and E).

Single nucleotide exchanges leading to an increased CAT expression (promoter up-mutations) may also help us to understand the importance of single nucleotides located in the promoter region. The influenza A virus studies revealed several complementary double mutants with an increased level of CAT expression (9, 10, 31). However, we found only one UUK promoter mutant (A6U) that displayed a 10% higher activity (pRF120; Fig. 1B and E) than the wild-type construct. Since this mutation is located in the spacer region between sites A and B (5′ arm), no further conclusions can be drawn at this point from this result.

The mutational analysis of the bulge region included single nucleotide substitutions as well as insertions and deletions. Single point mutations demonstrated that the type of each of the two nucleotides in the bulge is important (position 11 is a purine and position 12 is a pyrimidine). The insertion of the UG nucleotides in the 3′ promoter arm opposite to the 5′ bulge nucleotides (pRF104) generated a fully complementary promoter region of 17 bp in the panhandle. This mutant displayed a strong CAT activity (94% compared to the wild-type promoter). This is similar to the results obtained for the equivalent mutations in the influenza A virus promoter (construct pHL1140 in references 7 and 31). However, the deletion of one or both bulge nucleotides resulted in no detectable expression (7) or strongly decreased reporter gene expression in the influenza A virus study (43), whereas the UUK promoter could tolerate such deletions (Fig. 2C, pRF101 and pRF105). Therefore, deletions and insertions revealed that the bulge nucleotides are less important for the promoter activity than the proximal two recognition sites A and B. Since our system only allows for an assessment of the effect on transcription and replication, it cannot be excluded that the bulge nucleotides are important for the packaging process, as has been recently shown for influenza A virus (43). Further studies including the passaging of recombinant UUK viruses to fresh cell cultures with subsequent reporter analysis need to be carried out to address this question.

The mutational analysis of the UUK distal promoter element reveals surprisingly major differences compared to studies carried out on the influenza A virus (9, 10) and the RVF virus (37) promoter regions. In our UUK study neither single point substitutions nor changing of panhandle base pairs within the distal promoter element altered the resulting promoter activity. The distal element of the influenza A promoter, on the other hand, plays an important role during the viral polymerase interaction, either as a stabilizing double-stranded promoter element (10, 13, 14, 21) or as a regulatory element for viral mRNA transcription rates (12).

Studies with RVF demonstrated that the purine at position 13 at the 3′ promoter branch, which is highly conserved within the genus *Phlebovirus*, could not be changed into any other nucleotide without completely abolishing reporter gene expression (37). The UUK mutational analysis, however, showed no effect of any kind of substitution at position 13 (pRF184, G13A; pRF275, G13U; pRF276, G13C; Fig. 1E), suggesting that this position is not specifically recognized during the interaction between the viral polymerase and the UUK minigenome promoter.

To find out why some single point mutations totally abolished the promoter activity, we analyzed the synthesis of vRNA and cRNA. The point mutations could have affected different steps in the replication and transcription processes of the UUK minigenomes. The binding of the viral polymerase to the vRNA or the cRNA templates could be affected and could

thereby affect the efficiency of the template-dependent transcription and/or replication steps. Furthermore, the mutations could influence the cap-snatching or endonucleolytic cleavage and priming processes and thereby affect mRNA synthesis. We therefore estimated the amount of vRNA and cRNA in transfected cells by semiquantitative RT-PCR and calculated the vRNA/cRNA ratio. We did not attempt to determine the amount of mRNA, because the nondetectable levels of CAT activity indirectly indicated total absence of functional viral mRNAs. The vRNA/cRNA ratio of about 9:1 obtained for the wild-type promoter construct was expected, since synthesis of cRNA, a replicative intermediate, in most viral systems is substantially lower than that of vRNA synthesis. This result was similar to that obtained for influenza A virus, where a vRNA/cRNA ratio of 10:1 was demonstrated (30). However, only a minor influence on the vRNA/cRNA ratio could be detected for the mutants tested. This could mean that the mutations primarily affect transcription rather than replication. Further experiments will have to be carried out to demonstrate the exact mechanism by which the CAT-negative promoter mutants inhibit the transcription steps within the viral life cycle.

Based on the studies of the influenza A virus promoter elements, a novel corkscrew model was introduced to explain the role of the secondary structure of the influenza A virus promoter region (8, 9, 10, 25, 26). This model underscores the importance of intrastrand stem-loop structures for promoter function. Interestingly, nucleotides that are important for the promoter activity of influenza virus map within these loop parts. The loops presumably facilitate interaction with the viral polymerase complex (9, 10, 25, 26). Recent studies have confirmed that the corkscrew structure also plays an important role for the promoter activity of influenza C viruses (3) and thogoto viruses (24, 47). This suggests that the corkscrew is a common promoter structure for members of the *Orthomyxoviridae* (7).

Comparison of the promoter sequences of the five genera comprising the *Bunyaviridae* family revealed for members of four of the genera the possibility for intrastrand stem-loop structures within the proximal promoter region (Fig. 6), in conformity with the corkscrew model (7). This is also true for Tenuiviruses, which share many features common to *Bunyaviridae*. Interestingly, the only exceptions are the phleboviruses, for which no stem-loop structure(s) could be found. Our mutagenesis studies do not offer an obvious explanation for this unique feature of phleboviruses. Complementary double mutations at positions 4 and 8 that allowed for intrastrand base pairing and the potential to form a corkscrew configuration completely abolished promoter activity (unpublished data). Whether the lack of the potential to form a corkscrew configuration reflects an evolutionary divergence or a different mechanism for polymerase-promoter interaction must await further experimental analyses.

The reverse genetics system and the results presented here for UUK virus will hopefully be useful as a model for similar approaches in studies of the highly pathogenic members of the *Bunyaviridae* family.

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Commentary

Reverse genetics of negative-strand RNA viruses: Closing the circle

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The study of viruses and their interactions with host cells and organisms has benefited greatly from the ability to engineer specific mutations into viral genomes, a technique known as reverse genetics. Genome manipulations of DNA viruses, either by transfecting cells with plasmids encoding the viral genome (1) or by heterologous recombination of plasmids bearing viral sequences with the virus genome (2–4), were the first to be performed. Positive-strand RNA virus genome manipulation followed quickly, partly because the viral genome is also mRNA sense. Simply transfecting plasmids, or RNA transcribed from plasmids, containing the poliovirus genome into susceptible cells resulted in the recovery of infectious poliovirus (5, 6). The negative-strand RNA viruses include a number of human and animal pathogens such as influenza A, B, and C viruses, hantaviruses, Lassa virus, rabies virus, Ebola virus, Marburg virus, measles virus, canine distemper virus, rinderpest virus, respiratory syncytial virus, mumps virus, human parainfluenza virus types 1–4, and Nipah virus (which recently emerged in Malaysia, causing respiratory distress and encephalitis in pigs and humans). However, the genomes of the negative-strand RNA viruses have been less amenable to artificial manipulation for several reasons: (*i*) precise 5′ and 3′ ends are required for replication and packaging of the genomic RNA; (*ii*) the viral RNA polymerase is essential for transcribing both mRNA and complementary, positive-sense antigenome template RNA; and (*iii*) both genomic and antigenomic RNAs exist as viral ribonucleoprotein (RNP) complexes (reviewed in ref. 7). The segmented genomes of influenza viruses, bunyaviruses, and arenaviruses allowed some genetic manipulation through the isolation of reassortant viruses, but manipulation of the complete genome of segmented negative-strand RNA viruses has progressed slowly, hampered by the very fact that the genome is segmented.

In this issue of the *Proceedings*, Neumann and coworkers (8) have come full circle on recovering recombinant, segmented negative-strand RNA viruses with the production of influenza virus entirely from plasmid DNA and driven only by the host cell transcription and translation machinery. Coming nearly 10 years after the first published reports of influenza virus genome manipulation (9) and after another *Proceedings* article describing the generation of recombinant bunyaviruses wholly from cDNA by using a recombinant vaccinia virus-driven system (10), virologists finally have acquired the tools necessary to perform sophisticated and comprehensive investigations of the role of all influenza virus proteins and RNA elements in replication and pathogenesis.

The influenza virus RNPs, upon their release into the cytoplasm of an infected cell, enter the cell nucleus, and the influenza virus polymerase complex, consisting of the PA, PB1, and PB2 proteins, begins to transcribe the genomic RNA into mRNA and a positive-sense antigenome RNA that serves as the template for the production of genome RNA. Although influenza virus was the first negative-strand RNA virus to have individual virus genes replaced by artificially manipulated segments, the difficulty in dealing with a segmented RNA genome, as well as the use of labor-intensive and selection-dependent techniques to drive reverse genetics has hindered the application of this technology. Nonetheless, many important discoveries pertaining to individual influenza virus proteins as well as demonstrating the use of influenza virus to serve as a viral expression vector have been obtained by application of the existing reverse genetics technology (reviewed in refs. 7 and 11).

Neumann and coworkers (8) have established a system that conscripts the host cell into making the equivalent of newly released RNPs by cotransfecting eight plasmids encoding each of the influenza virus genomic RNA segments under control of the RNA polymerase type I (pol I) promoter and transcription terminator along with four plasmids encoding the polymerase complex proteins and nucleoprotein (NP) cDNAs under control of an RNA polymerase type II (pol II) promoter. Although the concept of cotransfecting multiple plasmids to reconstitute a biochemical activity was pioneered for studying herpes virus DNA replication (12), the daunting nature of this 12–17 plasmid transfection (a likely record for most plasmids transfected into one cell) still results in approximately 1 in 1,000 cells producing infectious virus. The lack of a helper influenza virus allows the virus from the initial transfection to be characterized immediately, thus limiting the chance of viruses containing reversions or second-site mutations from becoming significant contaminants. One can only speculate as to how quickly our knowledge of influenza virus will progress, now that every nucleotide of the viral genome can be mutated and engineered back into the genome, in nearly endless combinations with other mutations.

As with most important scientific advances, the work of Neumann and coworkers builds on a large body of experiments that have identified the basic requirements for replicating and packaging influenza virus RNA segments. The technique used first to introduce a new, artificial RNA segment into influenza virus (13) and refined subsequently to create influenza viruses containing neuraminidase (NA) proteins derived from plasmid cDNAs (9) relied on reconstitution of viral RNPs from *in vitro*-transcribed RNA and purified nucleocapsid proteins (Fig. 1). The protein-RNA complex was transfected into cells, followed by infection with a helper influenza virus. The application of a selection pressure against the helper virus facilitates the detection of progeny virus containing the plasmid DNA-derived RNA segment. Although a tour de force of molecular biology at the time, the technique requires the purification of large amounts of viral nucleocapsid proteins and is most efficient when a strong selection pressure can be applied against the helper virus.

The use of pol I transcripts to produce artificial influenza virus RNA segments was pioneered by Hobom and colleagues (14–16). Unlike the mRNA transcripts produced by pol II, the primary RNA transcripts synthesized by pol I are ribosomal RNAs that possess neither a 5′ cap structure nor a 3′ poly(A) tail. Zobel and coworkers (16) successfully produced artificial influenza virus RNA segments with precise 5′ and 3′ ends, and

The companion to this Commentary begins on page 9345.

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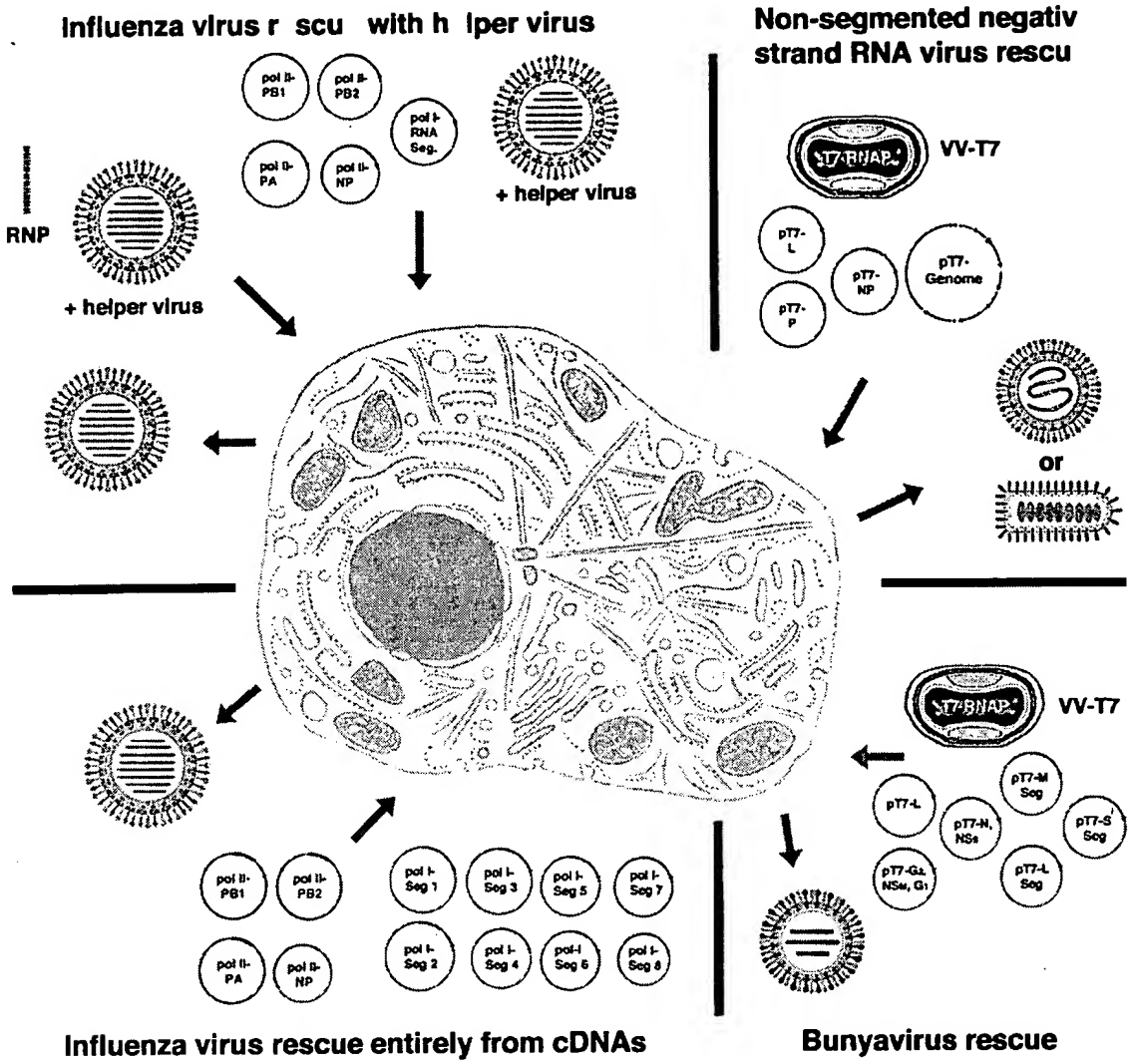

FIG. 1. Methods used to recover negative-strand RNA viruses from plasmid DNA. Several methodologies have been used to rescue negative-strand RNA viruses from plasmid-derived DNA. The initial replacement of individual RNA segments of influenza virus involved either (*i*) the *in vitro* reconstitution of RNPs or (*ii*) the *in vivo* assembly of RNPs after transfection of a cell with plasmids that use pol II promoters driving the expression of the PA, PB1, PB2, and NP proteins, and pol I promoters and terminators controlling viral genome synthesis. In either case, transfection was followed by infection with a helper influenza virus. Nonsegmented negative-strand virus rescue involves the transfection of plasmids encoding the viral P, N, and L proteins (and sometimes other viral proteins depending on the virus), as well as the viral antigenome, all under control of the bacteriophage T7 RNAP promoter. The T7 RNAP is provided by either infection with vv-T7, a recombinant vaccinia virus that expresses T7 RNAP or by transfecting into cell lines that stably express the protein. Bunyavirus rescue requires the transfection of plasmids encoding the three RNA segments in the antigenomic sense, along with three plasmids encoding the viral proteins, all under control of the T7 RNAP promoter. Influenza virus rescue entirely from plasmid DNA involves the transfection of plasmids encoding each of the eight RNA segments (under control of the pol I promoter and terminator) and plasmids encoding the four proteins that make up the polymerase complex (under control of the pol II promoter).

subsequent studies demonstrated that these genomic RNA constructs could be recognized and replicated by the influenza virus polymerase complex and packaged into progeny influenza viruses (14, 15). Pleschka and coworkers (17) used this technique to replace the viral RNA segment encoding the NA glycoprotein with a plasmid-based construct, showing the technique could substitute for RNP reconstitution in replacing single viral RNA segments. In addition, the artificial pol I transcript could be replicated and packaged into RNPs simply by cotransfecting plasmids encoding the PA, PB1, PB2, and NP proteins (17), shown previously to be the minimal proteins required to reconstitute influenza virus polymerase activity (18). The system described by Neumann and coworkers (8) represents the logical and important culmination of this body of work, finally resulting in the ability to manipulate every gene in the influenza virus genome.

Concurrent with efforts to perform reverse genetics with influenza virus, techniques to manipulate the genomes of nonsegmented negative-strand RNA viruses were being developed (reviewed in ref. 19). The task proved quite frustrating until Schnell and coworkers (20) made the somewhat counterintuitive, yet innovative, discovery that cotransfecting plasmids encoding the rabies virus L, P, and N protein cDNAs, as well as the viral antigenome, under control of the bacteriophage T7 RNA polymerase (T7 RNAP) promoter, into cells infected with a recombinant vaccinia virus expressing the bacteriophage T7 RNAP protein (vv-T7) (21) resulted in the recovery of recombinant rabies virus. The reverse genetics technique was quickly adapted by laboratories studying other nonsegmented negative-strand RNA viruses, resulting in the rescue of vesicular stomatitis virus (22, 23), measles virus (24), respiratory syncytial virus (25), Sendai virus (26, 27), rinder-

pest virus (28), human parainfluenza virus 3 (29, 30), simian virus 5 (31), and Newcastle disease virus (32). In some of these studies, rescue also has been accomplished by using the genome sense RNA (27, 30). Some refinements to the original technique have been made, such as the use of stably transfected cell lines expressing the T7 RNAP (in lieu of vv-T7 infection), or one or more of the viral proteins required for genome replication (24).

Several technical aspects of plasmid-based rescue of influenza virus as described by Neumann and coworkers (8) should be explored further. The number of recombinant viruses rescued can be increased nearly 10-fold by including plasmids encoding the hemagglutinin, NA, M_1, M_2, and NS_2 proteins under control of the pol II promoter in the transfection. The simplest explanation for this would be that the presence of the other influenza virus structural proteins allows packaging of RNPs at an earlier time posttransfection, resulting in more progeny virus being released. Alternatively, the M_1 and NS_2 (suggested to be renamed the nuclear export protein, NEP) proteins are known to function in modulating the import and export of RNPs from the nucleus of influenza virus-infected cells (33, 34), and the presence of these proteins alone may increase the amount of cytoplasmic RNPs available for packaging into progeny virions. Also, the production of spliced mRNAs from RNA segments 7 and 8 depends only on host cell factors, and the relative amounts of the spliced mRNAs present vary from cell to cell type (35, 36), which may affect virus recovery. The major difference between plasmid-based rescue of most nonsegmented negative-strand RNA viruses and influenza viruses involves the use of plasmids expressing antigenome or genome-sense RNA transcripts, respectively. Although the virus rescue efficiency of the plasmid-derived influenza virus is quite good, the use of an antigenomic plasmid may increase efficiency even more. The application of pol I-mediated expression of nonsegmented and other segmented negative-strand RNA virus genomes has yet to be explored but the success of Neumann and coworkers will no doubt result in a flurry of activity. However, although influenza virus has evolved to replicate in the nucleus and to exploit the cell-splicing machinery, for other RNA viruses that replicate in the cytoplasm, successful use of the pol I recovery system will depend on the absence of cryptic splicing signals in RNA transcripts.

Plasmid-based recovery of influenza virus allows investigation of aspects of the influenza virus life cycle that are known to involve multiple RNA segments, such as the neurovirulence of influenza A/WSN/33 in mice (reviewed in ref. 37) or viral polymerase functions. The engineering of influenza virus vaccines also should be improved quickly, because nucleotide changes correlating with attenuating or temperature-sensitive phenotypes now can be specifically identified and introduced in various combinations to produce new potential vaccines. If a properly attenuated genetic background can be constructed independent of the hemagglutinin and NA genes, the time needed to generate new vaccine viruses should be reduced to days rather than weeks. The ability of influenza virus to package additional RNA segments also may allow the virus to be used as a vector for delivering proteins to cells for therapeutic purposes, although the stability of these viruses needs to be investigated further.

Along with technological advances such as the ability to recover "designer" viruses, the relative ease of constructing influenza virus genes from synthetic oligonucleotides (38) and the sequencing of genes from "extinct" influenza virus strains such as the highly virulent 1918 Spanish influenza virus strain (39), comes a responsibility to avoid the construction of viruses that may pose a public health hazard. Restraint should be practiced, especially when dealing with factors associated with increased virulence, such as hemagglutinin proteins with multibasic cleavage sites (reviewed in ref. 40), or in the use of NA subtypes known to confer trypsin-independent cleavage of hemagglutinin (41).

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Differential Effect of Nucleotide Substitutions in the 3′ Arm of the Influenza A Virus vRNA Promoter on Transcription/Replication by Avian and Human Polymerase Complexes Is Related to the Nature of PB2 Amino Acid 627

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Using a genetic system that allows the *in vivo* reconstitution of active ribonucleoproteins, the ability to ensure transcription/replication of a viral-like reporter RNA harboring the $G_3 \rightarrow A_3$, $U_5 \rightarrow C_5$, and $C_8 \rightarrow U_8$ mutations (triple 3-5-8 mutations) in the 3′ arm of the promoter was examined with core proteins from human or avian strains of influenza A viruses. The efficiency of transcription/replication of the viral-like RNA with the triple 3-5-8 mutations in COS-1 cells was found to be slightly decreased as compared to the wild-type RNA when the polymerase was derived from a human virus. In contrast, it was found to be considerably increased when the polymerase was derived from an avian virus, in agreement with published observations using the avian A/FPV/Bratislava virus (G. Neumann and G. Hobom, 1995, *J. Gen. Virol.* 76, 1709–1717). This increase could be attributed to the compensation of the defect in transcription/replication activity in the COS-1 mammalian cell line due to the presence of a glutamic acid at PB2 residue 627, characteristic of avian strains of influenza viruses. Our results thus suggest that PB2 and/or cellular proteins interacting with PB2 could be involved in RNA conformational changes during the process of transcription/replication.

INTRODUCTION

The genome of influenza A viruses consists of eight molecules of single-stranded RNA of negative polarity. The viral RNAs (vRNAs) are packed with the nucleoprotein (NP) and associated with the three subunits of the polymerase complex (PB1, PB2, and PA) to form ribonucleoproteins (RNPs). Once the RNPs have entered the nucleus of the infected cells, they undergo transcription and replication (for a review, see Lamb and Krug, 2001). The three subunits of the polymerase and the NP are the minimum set of proteins required for both processes, although the molecular mechanisms involved are clearly different. The initiation of transcription involves a cap-stealing mechanism, by which 5′-capped oligonucleotides derived from cellular mRNA are used as primers and elongated by the viral polymerase (Bouloy *et al.*, 1978; Plotch *et al.*, 1981). Termination and polyadenylation occur at a stretch of five to seven U residues close to the 5′ end of the template (Luo *et al.*, 1991; Poon *et al.*, 1999). In the replication process, full-length positive-stranded RNAs complementary to the vRNAs (cRNAs) are produced, which in turn serve as templates for amplification of the vRNAs. Initiation of the synthesis of cRNAs and vRNAs is primer-independent, and antitermination occurs at the poly(U) sequence during cRNA synthesis (Hay, 1998). Although there is a recent article about cRNA-promoted transcription of mRNAs (Azzeh *et al.*, 2001), many experimental results indicate that it is very inefficient as compared to vRNA-promoted transcription (Cianci *et al.*, 1995; Honda *et al.*, 2001; Leahy *et al.*, 2002).

Genomic RNA segments of influenza A viruses are characterized by highly conserved sequences of 13 nt at their 5′ end and 12 nt at their 3′ end. Nucleotides 1′–16′ at the 5′ end and 1–15 at the 3′ end (i.e., the conserved sequences plus three additional nucleotides specific of the various segments) have been shown to contain the necessary signals for transcription, replication, and packaging of the genome segments (Fodor *et al.*, 1994; Luytjes *et al.*, 1989; Neumann *et al.*, 1994). Both the 5′ and the 3′ end are required for initiation of transcription in a stepwise interaction with the PB1 subunit of the polymerase complex (Li *et al.*, 1998; Tiley *et al.*, 1994), and thus they constitute the 5′ and 3′ arm of the vRNA promoter, respectively. The corresponding complementary sequences of the cRNA control vRNA synthesis and together form the cRNA promoter (Azzeh *et al.*, 2001). In addition, nonconserved sequences of the noncoding regions (NCR) of the genomic segments were found to modulate the efficiency of transcription/replication (Bergmann and Muster, 1996; Zheng *et al.*, 1996).

Several models have been proposed for the secondary structure of the influenza virus vRNA and cRNA promot-

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ers. The 5′ and 3′ sequences show partial inverted complementarity to each other. Chemical RNA modification experiments of native RNPs have indicated that in the presence of the polymerase complex, the vRNA 5′ and 3′ ends form a double-stranded "panhandle" structure; however, if not stabilized by the polymerase, base-pairing is not observed (Klumpp *et al.*, 1997). *In vivo* mutational analysis of viral-like reporter RNAs have led to the model of a "corkscrew" conformation for the vRNA promoter (Flick *et al.*, 1996). The features of the corkscrew conformation are two short proximal stem-loop structures, followed by a distal double-stranded RNA stem four to seven base pairs in length depending on the genomic segment. The proximal stem-loop elements and the distal stem are separated by a flexible hinge formed by a single unpaired nucleotide on the 5′ arm. In *in vitro* experiments, the presence of a stem-loop at both the 5′ and the 3′ end of the vRNA appears to be required for endonuclease activity (Leahy *et al.*, 2001a,b), while polyadenylation of the mRNAs requires the presence of a stem-loop at the 5′ but not at the 3′ end (Pritlove *et al.*, 1999). Detailed mutational analysis of the 9 nt involved in the formation of stem-loops at the 3′ and 5′ ends of the vRNA indicated that at some positions (e.g., nt 3 and 8 of either end) base-pairing within the promoter is critical with regard to polymerase activity rather than the identity of the nucleotides themselves; while at other positions (e.g., nt 2 and 9 of either end, nt 5 of the 5′ end), the nature of the nucleotide is important, probably because they represent positions of direct RNA–protein interactions (Flick and Hobom, 1999; Leahy *et al.*, 2001b).

A puzzling observation by Neumann and Hobom is that, in cells infected with the A/FPV/Bratislava virus, a viral-like RNA harboring combined $G_3 \rightarrow A_3$, $U_5 \rightarrow C_5$, and $C_8 \rightarrow U_8$ mutations (triple 3-5-8 mutations) in the stem-loop structure of the 3′ end undergoes transcription/replication with considerably enhanced efficiency as compared to the wild-type viral-like RNA (Neumann and Hobom, 1995). The aim of the present study was to examine whether the observation of Neumann and Hobom could be extended to other influenza A viruses or whether it was dependent on the avian vs human origin of the virus. We made use of a plasmid-based system described by Pleschka *et al.* (1996) for the *in vivo* reconstitution of functional RNPs, upon expression of wild-type or mutant viral-like RNAs together with the four core proteins (PB1, PB2, PA, and NP) derived from five different strains of human or avian viruses. We found that in COS-1 cells the triple 3-5-8 mutations increased transcription/replication of the vRNA only in the presence of polymerase complexes of avian origin. This increase was shown to correspond to the compensation of the defect in transcription/replication activity due to the presence of a glutamic acid at PB2 residue 627, characteristic of avian strains of influenza viruses (Naffakh *et al.*, 2000; Subbarao *et al.*, 1993). Our results are discussed with regard to the current models for molecular interactions and conformational changes that may take place within the RNP during the process of transcription/replication.

RESULTS

Variable effects of combined $G_3 \rightarrow A_3$, $U_5 \rightarrow C_5$, and $C_8 \rightarrow U_8$ mutations in the 3′ end of a viral-like RNA on the levels of transcription/replication in COS-1 cells, depending on the origin of the polymerase complex

The effects of combined $G_3 \rightarrow A_3$, $U_5 \rightarrow C_5$, and $C_8 \rightarrow U_8$ mutations (triple 3-5-8 mutations) in the proximal 3′ NCR of a type A viral-like CAT reporter RNA were studied in the presence of various polymerase complexes. The three mutations were introduced into the previously described pA/PRCAT(−) plasmid (Crescenzo-Chaigne *et al.*, 1999) to generate pA/PRCATmu3-5-8(−), as described under Materials and Methods. Plasmids pA/PRCAT(−) and pA/PRCATmu3-5-8(−) direct the synthesis of a wild-type (wt) and a mutated viral-like RNA, respectively (Fig. 1). Both RNAs are likely to adopt a corkscrew conformation (Flick and Hobom, 1999; Flick *et al.*, 1996) with a six base-pair double-stranded distal element (Fig. 1). They are analogous to the viral-like RNAs derived from the pHL926 and pHL1104 plasmids described by Neumann and Hobom (1995), except for the fact that the latter are predicted to form a four base-pair double-stranded distal element. To allow for direct comparison of our experiments with those from Neumann and Hobom, plasmids pA/PRCAT/NH(−) and pA/PRCAT/NHmu3-5-8(−) were generated by introducing both the $A_{14} \rightarrow C_{14}$ and the $C_{15} \rightarrow U_{15}$ mutations in pA/PRCAT(−) and pA/PRCATmu3-5-8(−), respectively. Thus, in the corresponding vRNAs, the length of the double-stranded distal element was reduced to 4 bp according to the corkscrew conformation model (Fig. 1). In the panhandle conformation model, the terminal nucleotides of the 5′ and 3′ NCRs are predicted to be only partially base-paired in the wt vRNAs derived from pA/PRCAT(−) or pA/PRCAT/NH(−) and to form a nine base-pair double strand in the mutant vRNAs derived from pA/PRCATmu3-5-8(−) or pA/PRCAT/NHmu3-5-8(−) (Fig. 1).

We compared the efficiency with which the four types of RNA templates underwent transcription/replication in the presence of *in vivo* reconstituted polymerase complexes derived from several human (A/PR/8/34, A/Victoria/3/75, and A/Paris/908/97) or avian (A/Mallard/NY/6758/78 and A/FPV/Rostock/34) type A influenza viruses. In COS-1 cells transfected with the pA/PRCAT(−) plasmid, the highest levels of CAT expression (in the range of 200-1000 ng per milliliter of cell extract prepared 24 h posttransfection), were achieved when A/PR/8/34 (PR8), A/Paris/908/97 (P908), or A/Victoria/3/75 (VIC) polymerase complexes were reconstituted, whereas reduced levels were observed for the A/FPV/Rostock/34 (FPV)

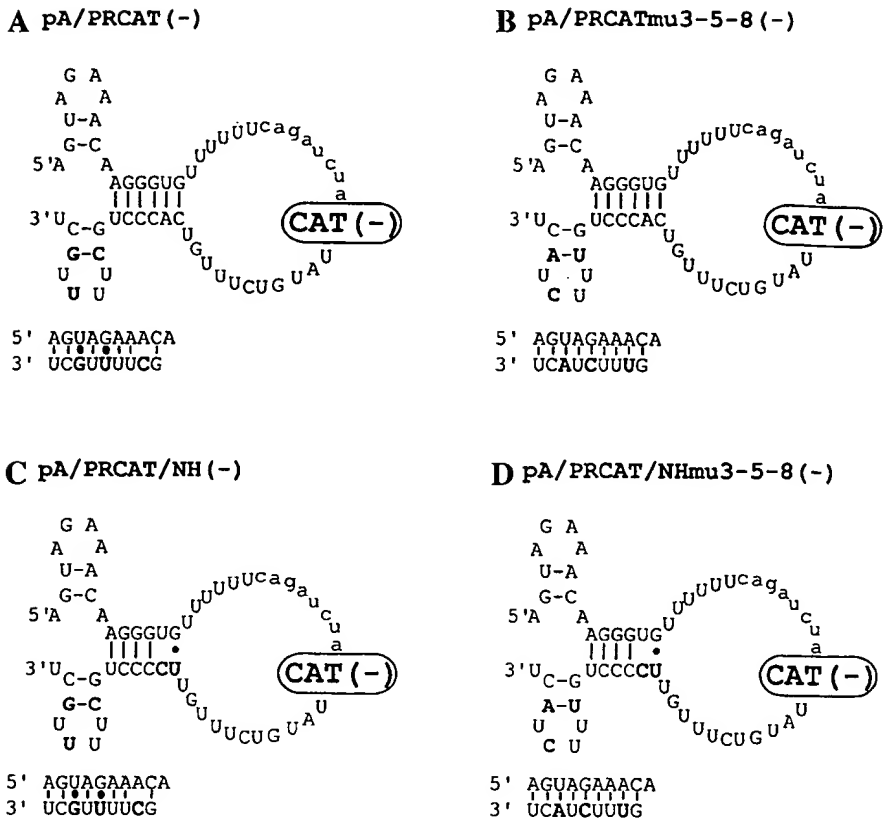

FIG. 1. Nucleotide sequence and predicted "corkscrew" and "panhandle" conformation of the minus (−)-sense model RNA templates. The nucleotide sequence of the extremities of the model RNA transcribed from pA/PRCAT(−) is the same as in the NS genomic segment of A/WSN/33 (uppercase). Unrelated nonviral sequences are shown in lowercase. The nucleotides mutated in pA/PRCAT(−)-derived plasmids are shown in bold. The predicted structures are as suggested by Flick *et al.* (1996).

and A/Mallard/NY/6758/78 (MAL) complexes (about 30 and 10 ng/ml of CAT, respectively) (Fig. 2A, black bars). The levels of CAT measured with the pA/PRCAT/NH(−)-derived RNA template were reduced two- to fourfold as compared to pA/PRCAT(−) (Fig. 2A, gray bars), in agreement with previously published observations which suggested that the length of the double-stranded distal element was not critical with regard to the efficiency of transcription/replication of type A vRNAs (Lee and Seong, 1998b). To examine the effects of the presence of the triple 3-5-8 mutations on the RNA templates, CAT levels were measured in cells transfected with pA/PRCATmu3-5-8(−) and expressed as a percentage of those measured with pA/PRCAT(−) (Fig. 2B, black bars); similarly, CAT levels measured with pA/PRCAT/NHmu3-5-8(−) and pA/PRCAT/NH(−) were compared (Fig. 2B, gray bars). At 24 h posttransfection, the "promoter-up" effect described by Neumann and Hobom (i.e., increased levels of transcription/replication of vRNAs harboring the triple 3-5-8 mutations; Neumann and Hobom, 1995) was observed only in cells expressing the FPV or the MAL complex (Fig. 2B). Indeed, CAT levels measured with the mutated viral-like RNAs (either with a 4-bp or a 6-bp double-stranded distal element) were about threefold (for FPV) and 30-fold (for MAL) higher than those obtained with the corresponding wt RNAs. In contrast, in cells expressing the PR8, P908, or VIC complexes, CAT levels measured with the pA/PRCATmu3-5-8(−)- and pA/PRCAT/NHmu3-5-8(−)-derived RNAs were either similar (for PR8 and P908) or 5- to 10-fold lower (for VIC) than those measured with the pA/PRCAT(−)- and pA/PRCAT/NH(−)-derived RNAs.

To understand better how the mutations in the 3′ NCR did interfere with the transcription/replication process, a kinetic analysis was performed using the pA/PRCAT(−) or pA/PRCATmu3-5-8(−) plasmids, as well as the corresponding pA/PRCAT(+) and pA/PRCATmu3-5-8(+) plasmids that encode (+) sense model RNA templates with a wt of mutated 5′ NCR, respectively. Each type of RNA template was coexpressed with either the P908-, the PR8-, the MAL-, or the FPV-derived complex, and the levels of CAT were measured in cell extracts at 18, 24, and 48 h posttransfection. As shown in Fig. 3A, in the presence of the P908 complex, the kinetics of accumulation of CAT protein were very similar with all four RNA templates. A rapid increase was observed up to 18 h posttransfection, followed by a slower increase between 18 and 24 h posttransfection and a plateau from 24 to 48 h posttransfection. The levels of CAT measured with the mutated RNA templates (hatched lines) were in the

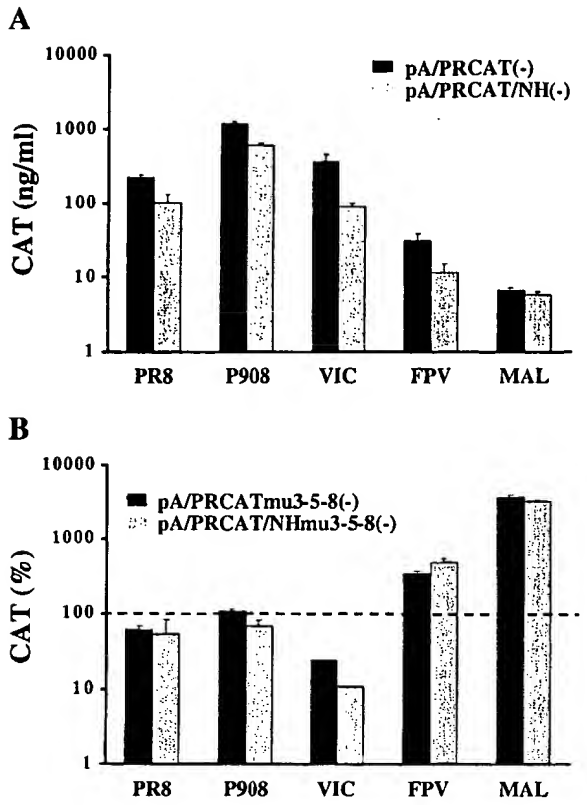

FIG. 2. Efficiency of transcription/replication of the (−)-sense model RNA templates in the presence of polymerase complexes of various origins. COS-1 cells were transfected with plasmids pA/PRCAT(−), pA/PRCATmu3-5-8(−), pA/PRCAT/NH(−), or pA/PRCAT/NHmu3-5-8(−), together with the four pHMG plasmids encoding the core proteins derived from PR8, P908, VIC, FPV, or MAL viruses. At 24 h posttransfection, cell extracts were prepared and tested for the levels of CAT as described under Materials and Methods. (A) For a given polymerase complex, CAT concentrations measured with pA/PRCAT(−) (black bars) were compared to those measured with pA/PRCAT/NH(−) (gray bars). (B) For a given polymerase complex, the CAT levels measured with pA/PRCATmu3-5-8(−) (black bars) were compared to and expressed as a percentage of those measured with pA/PRCAT(−) (100%, dotted line), and the CAT levels measured with pA/PRCAT/NHmu3-5-8(−) (gray bars) were compared to and expressed as a percentage of those measured with pA/PRCAT/NH(−) (100%, dotted line). The results are expressed as the mean ± standard deviation (SD) of duplicate samples from one representative experiment of three independent experiments.

same range as those measured with the wt templates (solid lines) at 18 and 24 h posttransfection, in agreement with the results shown in Fig. 2B, and appeared to be three- to fivefold lower at 48 h posttransfection. Very similar results were obtained with the PR8 complex (data not shown). With the MAL complex, when the wt RNA templates were used, the kinetics of accumulation of CAT were delayed as compared to that observed with the P908 complex: although CAT levels appeared higher with the (+)-sense than with the (−)-sense wt template, in both cases CAT levels remained low up to 18 h and increased rapidly between 18 and 48 h posttransfection (Fig. 3B, solid lines). Strikingly, when the mutated RNA templates were used, the kinetics of accumulation of CAT in the presence of MAL proteins were similar to that observed in the presence of P908 proteins, although CAT levels remained lower overall (Fig. 3B, hatched lines).

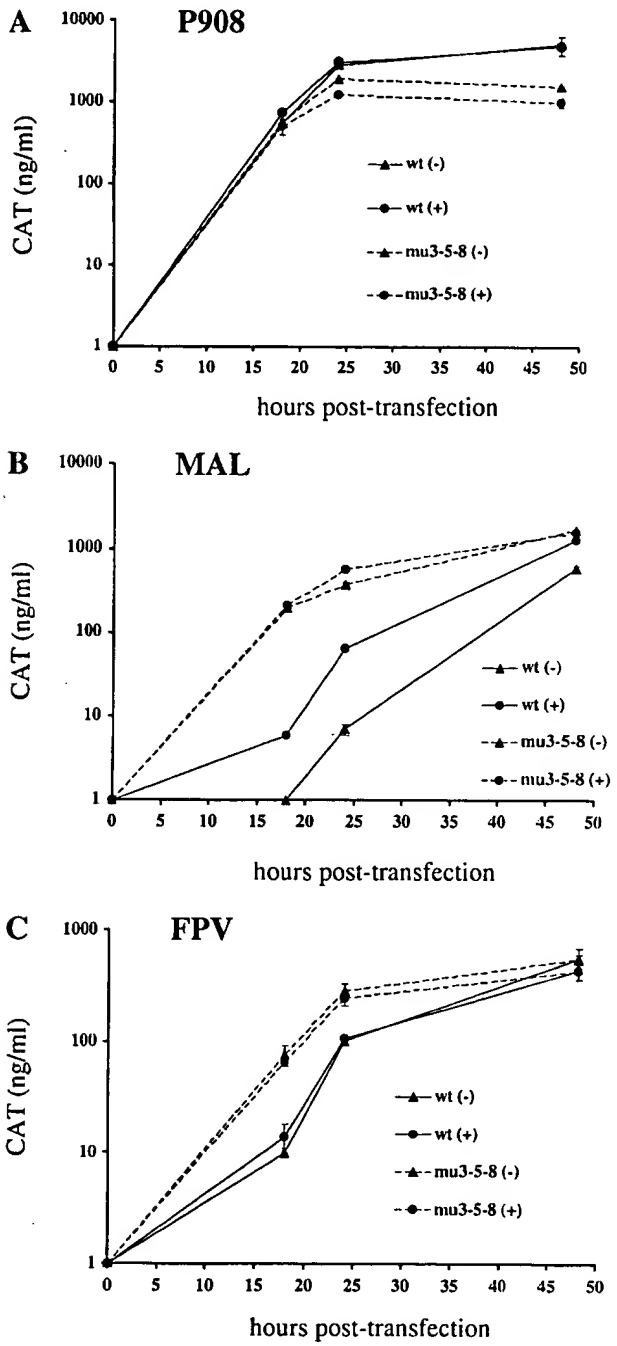

FIG. 3. Effect of the triple 3-5-8 mutations on the kinetics of transcription/replication of (−)- and (+)-sense model RNA templates in the presence of (A) P908-, (B) MAL-, or (C) FPV-derived polymerase complexes. COS-1 cells were transfected with the four pHMG plasmids encoding the core proteins together with either plasmids pA/PRCAT(−) (triangles, solid lines), pA/PRCAT(+) (circles, solid lines), pA/PRCATmu3-5-8(−) (triangles, broken lines), or pA/PRCATmu3-5-8(+) (circles, broken lines). At various times posttransfection, cell extracts were prepared and tested for the levels of CAT as described under Materials and Methods. The results are expressed as the mean ± SD of duplicate samples from one representative experiment of two.

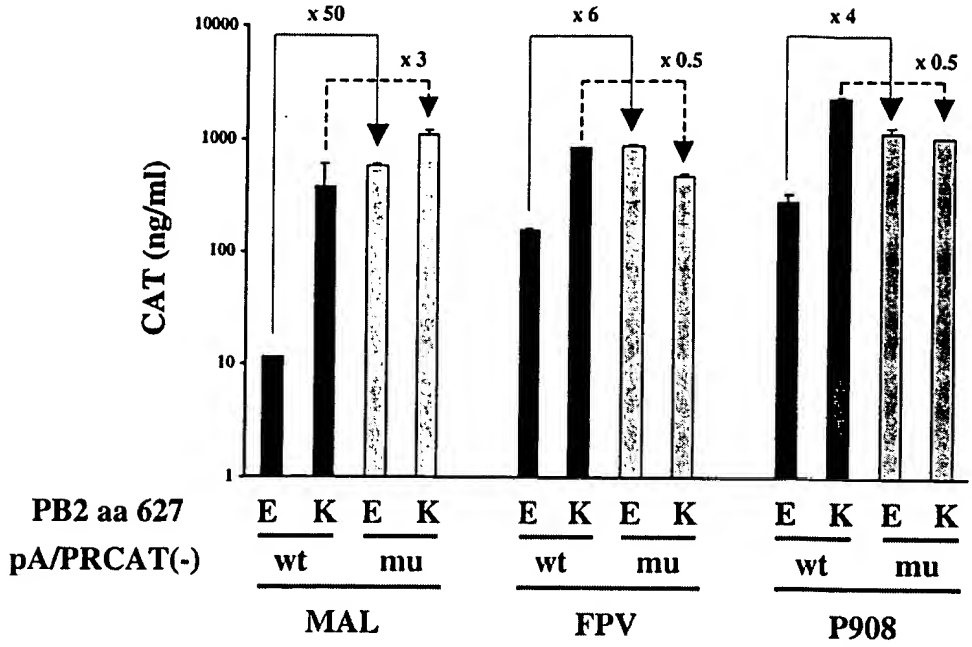

FIG. 4. Effect of the nature of PB2 amino acid 627 on the transcription/replication of wild-type or mutant (−)-sense model RNA templates. COS-1 cells were transfected with either plasmid pA/PRCAT(−) (wt, black bars) or pA/PRCATmu3-5-8(−) (mu, gray bars) together with the four pHMG plasmids encoding the core proteins derived from MAL, FPV, or P908 viruses. The pHMG-PB2 plasmids encoded PB2 proteins with either a glutamic acid (E) at residue 627 (wild-type PB2 for MAL and FPV-PB2 or mutant K627E-PB2 for P908) or a lysine (K) at residue 627 (mutant E627K-PB2 for MAL and FPV or wild-type PB2 for P908). At 24 h posttransfection, cell extracts were prepared and tested for the levels of CAT as described under Materials and Methods. Arrows indicate the effect of the triple 3-5-8 mutations on complexes with a glutamic acid (solid arrows) or a lysine (broken arrows) at residue 627 of PB2. The results are expressed as the mean ± SD of duplicate samples from one experiment representative of two.

The promoter-up effect of the mutations in pA/PRCATmu3-5-8(−) in the presence of the MAL complex was much more pronounced at 18 h (about 200-fold) than at 24 h (about 50-fold), or at 48 h posttransfection (threefold). Similarly, the promoter-up effect of the mutations in pA/PRCATmu3-5-8(+) was the highest at 18 h posttransfection (40-fold). The results obtained with the FPV complex were very similar to those observed with the MAL complex, although the differences between the CAT levels measured with the wt and mutated templates were lower (about sevenfold at 18 h, and threefold at 24 h posttransfection) (Fig. 3C). On the whole, these observations suggested that the presence of the triple 3-5-8 mutations in the 3′ NCR of the vRNA and/or the presence of the corresponding mutations in the 5′ NCR of the cRNA compensated for a functional defect of the MAL or FPV complex in transcription/replication of the wt viral-like RNA in COS-1 cells. This compensatory effect could operate at the level of recognition, amplification, and/or transcription of the RNA.

The triple 3-5-8 mutations in the 3′ end of a viral-like RNA compensate the defect in transcription/replication in COS-1 cells due to the presence of a glutamic acid at residue 627 of PB2

We have previously published that the efficiency with which the MAL or FPV complexes ensure transcription/replication of a wt viral-like RNA in COS-1 cells is enhanced if a MAL-PB2 or FPV-PB2 protein with a Glu → Lys mutation at residue 627 is expressed instead of the wt PB2 (Naffakh *et al.*, 2000). Here we asked whether the defect linked to residue Glu627 of PB2-MAL was compensated by the presence of the triple 3-5-8 mutations in the 3′ end of the vRNA. Thus we examined whether the efficiency with which the pA/PRCATmu3-5-8(−)-derived RNA underwent transcription/replication in COS-1 cells was still determined by the nature of PB2 residue 627. In addition to the plasmids encoding the E627K-MAL-PB2 and E627K-FPV-PB2 mutant proteins, which have been previously described (Naffakh *et al.*, 2000), we generated a plasmid encoding a P908-PB2 protein with a Lys → Glu substitution at position 627 (K627E-P908-PB2). The transcription/replication activity of MAL, FPV, and P908 complexes reconstituted with either a wild-type or a mutant PB2 protein was tested, using pA/PRCAT(−) or pA/PRCATmu3-5-8(−) as a template for synthesis of the viral-like RNA. The CAT levels measured at 24 h posttransfection are shown in Fig. 4. Using pA/PRCAT(−) as a template (black bars), CAT levels were 32-fold higher in cells expressing E627K-MAL-PB2 (385 ng/ml) than cells expressing the wt MAL-PB2 (12 ng/ml), fivefold higher in cells expressing E627K-FPV-PB2 (852 ng/ml) than cells expressing the wt FPV-PB2 (157 ng/ml), and fivefold lower in cells expressing K627E-P908-PB2 (327 ng/ml)

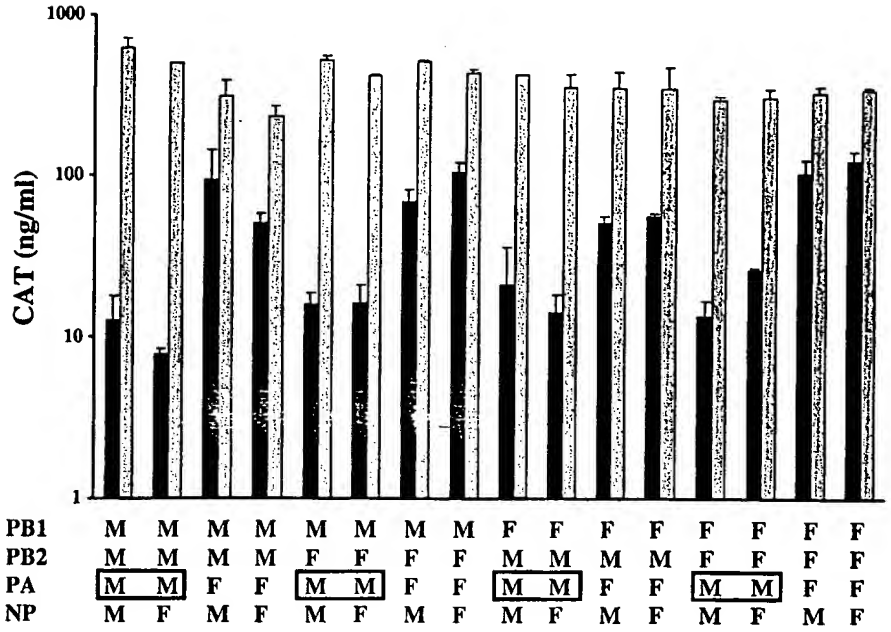

FIG. 5. Efficiency of transcription/replication of the wild-type or mutant (−)-sense model RNA templates in the presence of various combinations or MAL and FPV core proteins. COS-1 cells were transfected with plasmids pA/PRCAT(−) or pA/PRCATmu3-5-8(−), together with all 16 possible combinations between plasmids encoding the four MAL- and FPV-derived core proteins. At 24 h posttransfection, cell extracts were prepared and tested for the levels of CAT as described under Materials and Methods. For a given combination of core proteins, CAT concentrations measured with pA/PRCAT(−) (black bars) were compared to those measured with pA/PRCATmu3-5-8(−) (gray bars). The results are expressed as the mean ± SD of duplicate samples.

than cells expressing the wt P908-PB2 (1624 ng/ml). These observations were in agreement with our previously published data (Naffakh *et al.*, 2000) and confirmed that the nature of PB2 residue 627 is essential for the efficiency with which a polymerase complex ensures transcription/replication of a wt viral-like RNA in COS-1 cells. In contrast, when pA/PRCATmu3-5-8(−) was used as a template (gray bars), expressing the mutant instead of the wt PB2 proteins had little effect on CAT levels, which were in the range of 500-1000 ng/ml, whatever the origin of the polymerase complex and the nature of PB2 amino acid 627. As a consequence, the 50-fold promoter-up effect of the triple 3-5-8 mutations observed in the presence of the wild-type MAL complex (Fig. 4, solid arrow) was reduced to threefold when E627K-MAL-PB2 was expressed (Fig. 4, broken arrow). In the case of the FPV complex, a sixfold promoter-up effect was observed at 24 h with the wt (E627) FPV-PB2 but not with the E627K-FPV-PB2; conversely, a fourfold promoter-up effect was observed with the K627E-P908-PB2 protein but not with the wt (K627) P908-PB2 protein with which a twofold reduction was observed (Fig. 4).

The effects of the nature of PB2 amino acid 627 and of the triple 3-5-8 mutations were much more pronounced with the MAL than with the FPV complex, suggesting that they were modulated by some other molecular characteristics of each of the polymerase complexes. All 16 possible combinations between the core proteins of MAL and FPV were analyzed for the efficiency of CAT production in the presence of either the pA/PRCAT(−) or the pA/PRCATmu3-5-8(−) template. Whatever the combination of proteins, the levels of CAT measured in the presence of pA/PRCATmu3-5-8(−) were in the same range (250–650 ng/ml) (Fig. 5, gray bars). In contrast, the levels of CAT measured in the presence of pA/PRCAT(−) varied depending on the combination of core proteins: they were systematically lower when the PA subunit derived from MAL (10–20 ng/ml, instead of 50–120 ng/ml when PA derived from FPV) (Fig. 5, black bars), which resulted in a higher promoter-up effect of the 3-5-8 triple mutations. These results suggested that the difference observed between the FPV and MAL complexes was linked to the nature of the PA subunit, FPV-PA allowing a better efficiency of transcription/replication of the wt viral-like RNA in COS-1 cells than MAL-PA.

To examine whether the effects linked to the nature of PB2 amino acid 627 and to the nature of nucleotides 3, 5, and 8 at the 3′ end of the vRNA were similarly related to transcription and/or replication, we analyzed the RNA species involved these processes. COS-1 cells were transfected with plasmids encoding either MAL- or P908-derived core proteins together with the pA/PRCAT(−) or pA/PRCATmu3-5-8(−) plasmids. Total RNA was prepared at 24 h posttransfection, and 10 μg were used for the purification of polyadenylated RNAs. To detect vRNAs, total RNA (2 μg) was analyzed by Northern blotting and hybridization using a positive-sense CAT-specific ^{32}P-labeled riboprobe, as described under Materials and

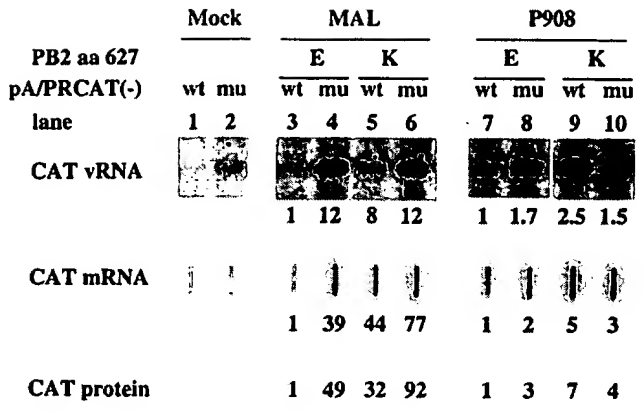

FIG. 6. Detection of CAT-specific RNAs in the transient transcription/replication assay. COS-1 cells were transfected with either plasmid pA/PRCAT(−) (wt) or pA/PRCATmu3-5-8 (−) (mu) together with the four pHMG plasmids encoding the core proteins derived from MAL (Lanes 3–6) or P908 viruses (Lanes 7–10), or with the empty pHMG plasmid (Mock, Lanes 1–2). The pHMG-PB2 plasmids encoded PB2 proteins with either a glutamic acid (E) or a lysine (K) at residue 627 as in Fig. 4. At 24 h posttransfection, total RNAs and poly(A)$^+$ RNAs were prepared and analyzed by Northern blotting followed by hybridization with a ^{32}P-labeled (+)-sense riboprobe, or by slot blotting followed by hybridization with a ^{32}P-labeled (−)-sense riboprobe, respectively, as described under Materials and Methods. The CAT-specific signals obtained by scanning the blots with a STORM820 optical scanner are shown. The amounts of CAT vRNA and mRNA were evaluated by using the Image Quant software and the corresponding amounts of CAT protein were measured by ELISA. After substraction of the values obtained in the mock samples, the values were expressed relative to those obtained with pA/PRCAT(−) (wt) in the presence of the wild-type PB2 for MAL (Lane 3), or mutant K627E-PB2 for P908 (Lane 7). The ratios are indicated below the corresponding CAT-specific RNA signals.

Methods. To detect mRNAs, polyadenylated RNAs were analyzed by slot blotting and hybridization using a negative-sense CAT-specific ^{32}P-labeled riboprobe, as described under Materials and Methods. Quantification of the hybridization signals indicated a good correlation between the levels of CAT mRNA and the protein (Fig. 6). Different levels of CAT vRNA were observed between the mock-transfected samples, the amount of CAT vRNA detected with the mutated template being repeatedly two- to threefold higher than the wt template (Fig. 6, Lane 2 compared to Lane 1). This difference could be due to an enhancement of the efficiency of ribozyme cleavage and/or to a higher stability of the CAT vRNAs synthesized from pA/PRCATmu3-5-8(−), because of the presence of the 3-5-8 triple mutations in the 3′ NCR. It is unlikely that it interfered with our analysis, because dose-response experiments indicated that CAT vRNA synthesized from pA/PRCAT (−) was in large excess (data not shown). In the presence of the MAL or P908 core proteins, significant variations of CAT mRNA and to a lesser extent of CAT vRNA were observed, when comparing the wt and mutant RNA templates, or the wt and mutant PB2 proteins. A Glu → Lys substitution in PB2 resulted in increased levels of vRNA and mRNA synthesized from the pA/PRCAT(−) template, albeit to a less significant extent with the P908 complex as compared to the MAL complex (Fig. 6, Lane 5 compared to Lane 3, and Lane 9 compared to Lane 7). Moreover, in the presence of a complex with a Glu627 residue in PB2, when pA/PRCATmu3-5-8(−) was used instead of pA/PRCAT(−), the levels of vRNA and mRNA were found to be increased, again to a much less significant extent with the P908 complex than the MAL complex (Fig. 6, Lane 4 compared to Lane 3, and Lane 8 compared to Lane 7). Consequently, when pA/PRCATmu3-5-8(−) was used as a template, a Glu → Lys substitution in PB2 had no significant effect on the levels of vRNA and mRNA synthesized (Fig. 6, Lane 6 compared to Lane 4, and Lane 10 compared to Lane 8). On the whole, these observations indicated that the transcription, and to a lesser extent the replication activity, was impaired when residue 627 of PB2 was a Glu, and that both defects were compensated by the presence of A_3, C_5, and U_8 nucleotides at the 3′ end of the vRNA. Furthermore, both the effects of residue 627 of PB2 and the effects of the triple 3-5-8 mutations were much more pronounced with the MAL than with the P908 complex.

The nature of nucleotides 3 and 8 at the 3′ end of a viral-like RNA is essential for the promoter-up effect of the triple 3-5-8 mutations observed in the presence of the MAL or FPV complex

To evaluate the respective contribution of mutations at nucleotides 3, 5, and 8 of the 3′ NCR to the promoter-up effect observed in the presence of the MAL or FPV complexes, two additional pA/PRCAT(−)-derived plasmids were generated, which contained either the $G_3 \rightarrow A_3$ and $C_8 \rightarrow U_8$ mutations (pA/PRCATmu3–8(−)) or the $U_5 \rightarrow C_5$ mutation alone (pA/PRCATmu5(−)). These plasmids were tested in the presence of P908-, MAL-, or FPV-derived complexes, in parallel with pA/PRCAT(−) and pA/PRCATmu3-5-8(−). The levels of CAT measured in cell extracts at 24 and 48 h posttransfection are shown in Fig. 7. Whatever the polymerase complex which was used, similar CAT levels were observed with plasmids pA/PRCATmu3-5-8(−) and pA/PRCATmu3–8(−) on the one hand (hatched lines), and with plasmids pA/PRCAT(−) and pA/PRCATmu5(−) on the other hand (solid lines). Similar results were obtained using the PR8-derived complex (data not shown). These observations indicated that the mutations at nt 3 and 8, but not nt 5 at the 3′ end of the viral-like RNA, were responsible for the promoter-up effect observed in the presence of the MAL or FPV complex. In agreement with the data from Flick *et al.* (1996), any single mutation at nucleotide 3 or 8, which disrupted the G_3-C_8 base pair predicted by the corkscrew model of vRNA, completely abolished transcription/replication of the RNA template (data not shown). When a U_3-A_8 or a C_3-G_8 base pair was restored

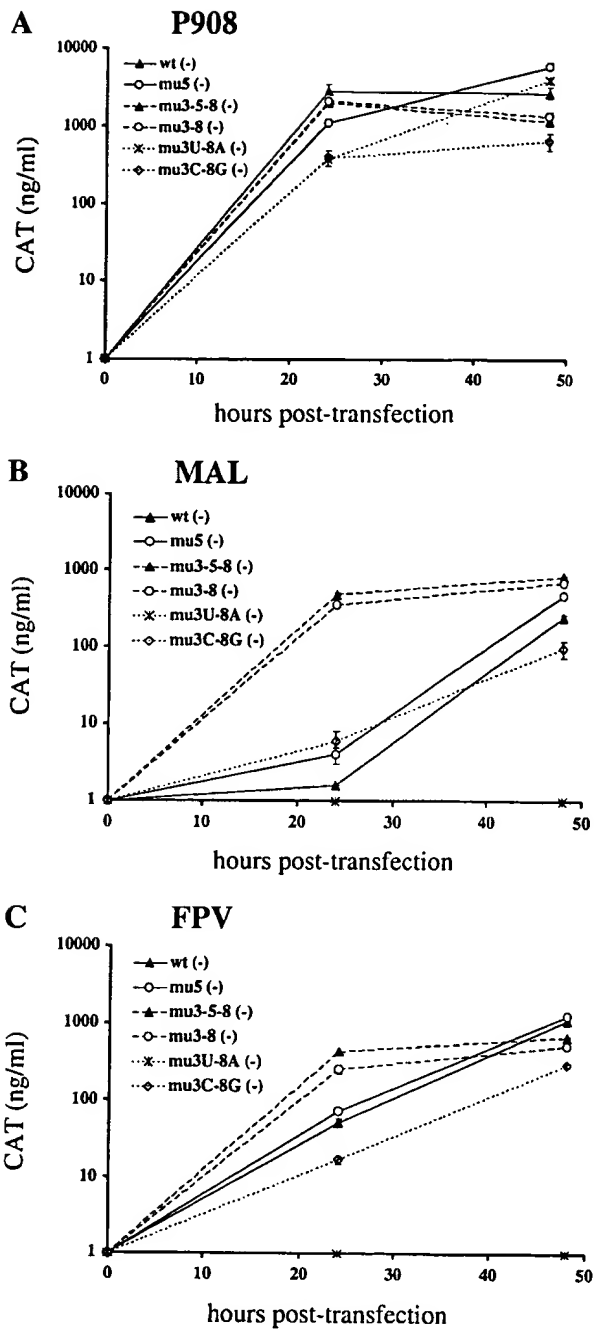

FIG. 7. Effect of single or double mutations as compared to the triple 3-5-8 mutations in the 3′ end of the (−)-sense model RNA template on transcription/replication in the presence of P908, MAL, or FPV-derived polymerase complexes. COS-1 cells were transfected with the four pHMG plasmids encoding the core proteins derived from (A) P908, (B) MAL, or (C) FPV viruses as indicated, together with either plasmid pA/PRCAT(−) (solid lines, closed triangles), pA/PRCATmu3-5-8(−) (broken lines, closed triangles), pA/PRCATmu5(−) (solid lines, open circles), pA/PRCATmu3-8(−) (broken lines, open circles), pA/PRCATmu3C-8G(−) (dotted lines, open squares), or pA/PRCATmu3U-8A(−) (dotted line, crosses). At 24 and 48 h posttransfection, cell extracts were prepared and tested for the levels of CAT as described under Materials and Methods. The results are expressed as the mean ± SD of duplicate samples from one representative experiment of two.

in the stem-loop (plasmids pA/PRCATmu3C-8G(−) and pA/PRCATmu3U-8A(−)), the levels of CAT measured in the presence of the P908 complex at 24 h posttransfection were about 10-fold lower than those observed with pA/PRCAT(−) or pA/PRCATmu3-5-8 (−) (Fig. 7A, dotted lines). In the presence of the MAL or FPV complexes, the levels of CAT measured at 24 h posttransfection with pA/PRCATmu3C-8G(−) were respectively about 100- and 25-fold lower than those measured with pA/PRCATmu3-5-8(−) (Figs. 7B and 7C, dotted lines, square symbols), while the levels of CAT measured with pA/PRCATmu3U-8A(−) were at background levels (Figs. 7B and 7C, dotted line, cross symbols). These very different levels of CAT observed depending on the nature of the predicted base pair in the 3′ stem-loop of the RNA templates, i.e., A_3-U_8 (mu3-5-8), U_3-A_8 (mu3U-8A), or C_3-G_8 (mu3C-8G), indicated that not only the base pairing between nt 3 and 8 but also the nature of nt 3 and 8 was essential for the promoter-up effect.

No sequence variations are found at nt 3, 5, or 8 in the 3′ and 5′ ends of the eight genomic RNA segments derived from the MAL and PR8 viruses upon sequencing

Based on all available sequences, the 12 nt at the 3′ end and the 13 nt at the 5′ end of influenza A genomic RNAs are highly conserved. However, the observation that a viral-like RNA with A_3, C_5, and U_8 nucleotides at the 3′ end could undergo efficient transcription/replication prompted us to sequence the 3′ and 5′ ends of the eight genomic segments derived from the MAL and PR8 viruses, to determine whether nt 3, 5, and 8 were conserved, or whether some sequence variation could occur and possibly be involved in controlling the segment-specific transcription/replication level. The 3′ and 5′ terminal nucleotides of PR8 segments had been determined by Desselberger *et al.* in 1980 (Desselberger *et al.*, 1980), but might have varied along the many subsequent passages of the virus. Data on the 3′ and 5′ terminal nucleotides of MAL segments were not available, and vRNA sequencing was performed on a batch of virus with the same passage history as for cloning of the MAL-PB1, -PB2, -PA, and -NP cDNAs used in our experiments. We optimized PCR-based protocols for direct sequencing of the 3′ and 5′ NCR, as described under Materials and Methods. Genomic RNA was extracted from the MAL and PR8 viruses and the sequence of the extremities of the eight genomic segments was determined. The 12 nt at the 3′ terminus and 13 nt at the 5′ terminus were found to be wild-type in all segments (data no shown). In agreement with published data (Desselberger *et al.*, 1980; Robertson, 1979), a unique variation was observed within these conserved sequences, U or C at position 4 of the 3′ end of the vRNA (Table 1). The distal double-stranded element in the corkscrew conformation model

TABLE 1

Sequence Characteristics of the Extremities of the Genomic Segments Derived from the MAL and PR8 Viruses as Compared to All Sequences Available in GenBank

	nt 4 of the 3′ end			Length of the ds distal element (bp)		
Segment	MAL	PR8	Other[a]	MAL	PR8	Other[a]
PB1	C	C	U/C	5	5	5
PB2	C	C	U/C	5	5	5
PA	C	C	U/C	7	7	7/6
HA (H1)	—	U	U	—	4	4
HA (H2)	U	—	U	4	—	4
NP	U	U	U	6	6	6
NA (N1)	—	C	U	—	3//4[b]	8
NA (N2)	U	—	U	6	—	6
M	U	C	U	6	6	6
NS	U	U	U	6	6	6

[a] All sequences available in GenBank for influenza A viruses other than MAL and PR8 were aligned, and the sequence consensus is indicated. The number of sequences available for the extremities was variable depending on the segment: 20 for PB1, 24 for PB2, 16 for PA, 11 for H1, 11 for H2, 88 for NP, 10 for N1, 9 for N2, 14 for M, and 21 for NS.

[b] Two elements of three and four base-paired nucleotides separated by a 1-nt gap.

(starting with base-pairing of nt 10 of the 3′ end and nt 11′ of the 5′ end) was found to be 4 to 8 bp long depending on the segments (Table 1). For PR8, the terminal sequences were found identical to those described by Desselberger *et al.* (1980), and the features that appear characteristic of PR8 as compared to all available sequences in the databases, i.e., a C at nt 4 in the 3′ end of segments NA and M, and a discontinuity in the distal stem of the NA segment, were confirmed (Table 1). On the whole, our data confirm the high conservation and stability of the terminal sequences of influenza A virus genomic segments.

DISCUSSION

Effect of the triple 3-5-8 mutations in the 3′ end of vRNA

It was previously observed by Neumann and Hobom that in murine B82 cells infected with the influenza virus A/FPV/Bratislava, a viral-like reporter RNA harboring triple 3-5-8 mutations in the 3′ end underwent transcription/replication more efficiently than a wild-type viral-like RNA, i.e., the mutations showed a promoter-up effect (Neumann and Hobom, 1995). Here, we report that this observation cannot be extended to all influenza A viruses. Indeed, using a plasmid-driven system for the *in vivo* reconstitution of functional ribonucleoproteins in COS-1 cells, we have observed that the triple mutations had a promoter-up effect when the polymerase complex was derived from the MAL or FPV viruses (avian viruses, as A/FPV/Bratislava), but not from the human PR8, VIC, or P908 viruses. In the presence of human-derived polymerase complexes, the levels of transcription/replication achieved with the mutated viral-like RNA were even lower (at least twofold) than the wild-type RNA, i.e., the mutations had a slight "promoter-down" effect. Similar results were obtained whether the RNA templates were designed to form a 6-bp-long (NS segment-like templates) or a 4-bp-long (HA segment-like templates) double-stranded distal element in the corkscrew conformation, which indicated that the effects of the triple 3-5-8 mutations were independent of the length of the distal stem. Moreover, experiments using RNA templates harboring various double or single mutations at nt 3, 5, 8 of the 3′ end indicated that the promoter-up and promoter-down effects observed with the RNA harboring the triple 3-5-8 mutations in the presence of avian- and human-derived complexes, respectively, were determined by the nature of nt 3 and 8, but not nt 5. Our finding, that not only base-pairing between nt 3 and 8 but also the nature of the nucleotide at these positions was critical for promoter activity, is contradictory with data from Flick and Hobom, which suggested that only base-pairing was critical in an HA segment-like vRNA (Flick and Hobom, 1999). Our observation that a viral-like RNA with a single U5 → C5 mutation in the 3′ end underwent transcription/replication as efficiently as the wild-type RNA is again in contradiction with data from Neumann and Hobom, who reported that this nucleotide exchange at position 5 completely abolished the promoter activity (Neumann and Hobom, 1995). Interestingly, Lee and Seong (1998a) as well as Bergmann and Muster (1995) reported that they were unable to rescue transfectant WSN viruses carrying stably the U5 → C5 mutation in the 3′ end of the NA vRNA. These apparent contradictions may result from the fact that the effects of the mutations were analyzed in the context of the noncoding sequences from different viral segments (i.e., NS in this article, HA (Neumann and Hobom, 1995; Flick and Hobom, 1999) or NA (Lee and Seong, 1998a; Bergmann and Muster, 1995) in the other studies). In addition, the discrepancies may be linked to the fact that in our exclusively plasmid-based experimental system, there is no expression of viral genes other than PB1, PB2, PA, and NP, whereas the results of the three teams cited above were obtained in the context of a viral infection.

In the course of a mutagenic analysis of the 3′ terminal sequence of the vRNA with regard to endonuclease activity of the polymerase complex of A/PR/8/34 virus, Leahy *et al.* observed that the identity of residue 5 did not affect endonuclease activity, whereas replacing the G-C base pair at position 3–8 in the stem-loop structure with an A-U base pair reduced endonuclease activity by about 50% (Leahy *et al.*, 2001a, 2002). This decrease in endonuclease activity could very well contribute to the two- to threefold decrease in the levels of CAT that we measured

with RNA templates harboring the double 3–8 or triple 3-5-8 mutations in the presence of PR8 or P908 complexes, as compared to the wild-type RNA.

Influence of the nature of residue 627 of PB2 on the effect of the triple 3-5-8 mutations

Our results indicate that the promoter-up effect of the triple 3-5-8 mutations observed with avian-derived complexes corresponds to the compensation of a defect of the transcription/replication activity of these complexes in COS-1 cells as compared to human-derived complexes. Indeed, the levels of transcription/replication measured at 24 h posttransfection with the MAL or FPV complex were 10- to 100-fold lower than those obtained with human-derived complexes when a wt viral-like RNA was used, whereas they were in the same range when the mutant viral-like RNA was used. Experiments using MAL-PB2 and FPV-PB2 mutants with a Glu627Lys substitution and a P908-PB2 mutant with a Lys627Glu substitution indicated that the transcription, and to a lesser extent the replication activity, was impaired when residue 627 of PB2 was a Glu (typical of avian viruses), and that both defects were compensated by the presence of the triple mutations at the 3′ end of the vRNA. Noticeably, both the effects of the nature of PB2 amino acid 627 and the effects of the triple 3-5-8 mutations were much more pronounced with the MAL than with the P908 complex and appeared intermediate with the FPV complex, which was probably linked to some other molecular characteristics of the FPV and P908 polymerase complexes, allowing a better transcription/replication activity in COS-1 cells than the MAL complex. Analysis of all 16 possible combinations between the core proteins of MAL and FPV indicated that the difference observed between the two complexes was linked to the PA subunit. The alignment of MAL- and FPV-PA with all other sequences available for PA reveals that several residues differ between MAL- and FPV-PA, but that none of them correspond to a position that differs between the human and avian lineages of influenza A viruses, which could have accounted for the intermediate behavior of the FPV complex as compared to MAL and P908 complexes.

It has been shown previously that PB2 and more specifically amino acid 627, as well as to a lesser extent PA, were determinants of the temperature sensitivity of the polymerase complex derived from avian viruses (Massin *et al.*, 2001), suggesting that the effect of the triple 3-5-8 mutations might be temperature-dependent. However, when the transcription/replication activities of avian (MAL, FPV) and human (P908) complexes were compared at 33, 37, and 40°C, the same differential effect of the triple mutations was observed at all temperatures (data not shown). Thus the hypothesis of a temperature-dependent effect seems unlikely.

Amino acid 627 of PB2 has recently been shown by Hatta *et al.* to be an essential determinant of the pathogenicity in mice of A(H5N1) influenza viruses which were transmitted from birds to humans in Hong Kong in 1997 (Hatta *et al.*, 2001). This was in agreement with previous studies on human/avian reassortant viruses which indicated that PB2 residue 627 was a host-range determinant of influenza A viruses (Subbarao *et al.*, 1993). Some of our previous data suggested that the mechanism by which the nature of PB2 amino acid 627 alters the efficiency of transcription/replication in mammalian (COS-1) cells is probably complex, relying on interactions of PB2 with viral and most importantly cellular proteins (Naffakh *et al.*, 2000). Our present data suggest that RNA–protein interactions are also involved, but the exact nature of these interactions and the mechanism by which they may be influenced by the presence of the triple 3-5-8 mutations in the 3′ end of the vRNA are still unclear, as little is known on the structure/function relationships for vRNA.

Structure dynamics of viral RNA and functional interactions with the polymerase complex

Flick *et al.* have suggested that during the process of transcription/replication, the vRNA could be in the panhandle conformation for initial interaction with the polymerase and then switch to a corkscrew conformation, the equivalent of an open complex structure (Flick *et al.*, 1996). The cRNA is also likely to adopt a panhandle and/or corkscrew conformation while interacting with the polymerase complex (Azzeh *et al.*, 2001). Leahy *et al.* have established that the identity of nt 5 in the 5′ arm of the cRNA promoter, together with the absence of a "hinge" A nt in the 5′ arm and the presence of a hinge U nt in the 3′ arm of the cRNA promoter in contrast to the vRNA promoter is the basis for the lack of activation of endonuclease activity of the complex upon binding to the cRNA (Leahy *et al.*, 2002). Finally, melting of the 3′ end of the vRNA and cRNA is probably required for the initiation of synthesis of (+)- and (−)-sense RNAs, respectively.

The triple 3-5-8 mutations in the 3′ end of the vRNA are likely to alter the structure of both the vRNAs and the cRNAs. First they are predicted to increase the stability of the terminal RNA duplex in the panhandle conformation model by restoring a complete Watson–Crick base pairing between the nine terminal nucleotides of the 3′ and 5′ ends. Interestingly, Bae *et al.* have recently described the structure of the vRNA promoter in the panhandle conformation using magnetic resonance spectroscopy and showed that there is a bending of the RNA duplex at position 4, which could lower the energy for unwinding and opening the helix (Bae *et al.*, 2001). They demonstrated that mutations in the 3′ end that restore a complete Watson–Crick base pairing within the RNA duplex suppress the bending of the helix, which could stabilize the panhandle structure. The triple 3-5-8 muta-

tions in the 3′ end of the vRNA are also predicted to lead to (i) a decreased stability of the 3′ arm of the vRNA promoter and of the 5′ arm of the cRNA promoter in the corkscrew conformation; and (ii) a sequence identity between the vRNA and cRNA promoters, except for the difference in the hinge localization which is maintained.

The most simple interpretation of our data is that the triple mutations compensate the inability of a polymerase complex with a Glu at PB2 amino acid 627 to stabilize the panhandle conformation and/or to melt stem-loop structures in the corkscrew conformation in COS-1 cells. This would suggest that PB2 and/or cellular proteins interacting with PB2 could be involved in RNA conformational changes during the process of transcription/replication. Some of the cellular proteins implicated could be helicases. Indeed, no helicase activity has been found associated with the polymerase complex of influenza A viruses, but Huarte *et al.* recently reported the detection of an interaction between the PA subunit of the complex and a protein containing an helicase domain (Huarte *et al.*, 2001). Further experiments involving both mammalian and avian cell lines could help in testing these hypotheses.

MATERIALS AND METHODS

Plasmids for the expression of viral proteins

Plasmids pHMG-PR8-PB1, -PB2, -PA, and -NP, which express the PB1, PB2, PA, and NP proteins, respectively, of influenza virus A/Puerto Rico/8/34 (PR8) under the control of the hydroxymethylglutaryl coenzyme A reductase (HMG) promoter were kindly provided by J. Pavlovic (Institut für Medizinische Virologie, Zurich, Switzerland). The analogous pHMG-derived plasmids encoding the PB1, PB2, PA, and NP proteins of A/Victoria/3/75, A/Mallard/NY/6758/78, and A/FPV/Rostock/34 viruses have been described previously (Naffakh *et al.*, 2000). Virus isolate A/Paris/908/97 (P908) was grown on MDCK cells. After four passages, viral genomic RNA was extracted from MDCK supernatant using the Trizol reagent (Gibco). Molecular cloning and sequencing of the cDNAs encoding P908-PB1, -PB2, -PA, and -NP were performed as described previously (Naffakh *et al.*, 2000). The corresponding sequences have been submitted to GenBank under the following Accession Nos.: AF483601, AF483602, AF483603, AF483604. The pHMG-MAL-PB2-E627K and pHMG-FPV-PB2-E627K plasmids, which express MAL and FPV-derived PB2 proteins mutated at residue 627, have been described in Naffakh *et al.*, 2000. Similarly, a plasmid allowing the expression of a P908-derived PB2 protein with a K627E mutation was generated using an overlap extension PCR protocol (Pogulis *et al.*, 1996). The pHMG-P908-PB2 plasmid was used as a template and oligonucleotides 5′-GCCGCTCCACCAGAACAAAGCAGGATG-3′ and 5′-CATCCTGCTTTGTTCTGGTGGAGCGGC-3′ were used as mutagenic primers. The sequence of the additional primers used for mutagenesis can be obtained from the authors upon request. The conditions of amplification were as described in Naffakh *et al.*, 2001. The fusion PCR product was digested with the *Hpa*I and *Kpn*I enzymes and subcloned into the pHMG-P908-PB2 plasmid at the *Hpa*I and *Kpn*I sites. Positive clones were sequenced using a Big Dye terminator sequencing kit (Perkin–Elmer) and analysis on an ABI prism 377 automated sequencer to assess the presence of the site-directed mutation and the absence of unanticipated nucleotide changes.

Plasmids for the expression of viral-like RNAs

The pA/PRCAT(−) and pA/PRCAT(+) plasmids, which direct the expression of an influenza virus-like RNA derived from the nonstructural (NS) segment of the A/WSN/33 virus, were described previously (Crescenzo-Chaigne *et al.*, 1999). They contain, inserted at the *Bbs*I site of vector plasmid pPR (Crescenzo-Chaigne *et al.*, 1999), the CAT gene sequences in an antisense (pA/PRCAT(−)) or sense (pA/PRCAT(+)) orientation flanked by the 5′ and 3′ extremities of the NS gene segment. Expression of the viral-like RNA is driven by a truncated human RNA *Pol*I promoter. The correct 3′ end is ensured by the use of the hepatitis delta virus ribozyme sequence.

The PCR-based protocol used to introduce mutations in the sequences of pA/PRCAT(−) corresponding to the 3′ end of the viral-like RNA has been described previously (Crescenzo-Chaigne and van der Werf, 2001). Primers 5′-AG*TAGAAA*CAGGGTGACAAAGAC-3′, 5′-AGCAAAAGCAGGG*GA*ACAAAGACATAATG-3′, and 5′-AG*TAGA*-*AA*CAGGGG*AA*CAAAGACATAATG-3′ were used with the primer 5′-AGTAGAAACAAGGGTGTTTTTTCAG-3′ for amplification of the CAT gene and NS noncoding sequences using pA/PRCAT(−) as a template, to generate the pA/PRCATmu3-5-8(−) and (+), pA/PRCAT/NH(−), and pA/PRCAT/NHmu3-5-8(−) plasmids, respectively. Similarly, primers 5′-AG*TAAAAA*CAGGGTGACAAAGAC-3′, 5′-AGCAG*AAGCAGGGTGACAAAGAC*-3′, 5′-AG*AAAAAT*C-AGGGTGACAAAGAC-3′ and 5′-AG*GAAAAC*CAGGGTGA-CAAAGAC-3′ were used to generate additional mutant plasmids pA/PRCATmu3-8(−), pA/PRCATmu5(−), pA/PRCATmu3U-8A(−), and pA/PRCATmu3C-8G(−). The amplified products were cloned at the *Bbs*I sites of pPR. The presence of the mutations in the positive clones was assessed by sequence determination as described above.

Transfections and CAT assays

COS-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Subconfluent monolayers (1.2×10^5 cells in 21-mm dishes) were transfected by using 5 μl FuGENE 6 (Roche) with 0.5 μg of either pA/PRCAT(−) or one of the pA/PRCAT(−)-de-

rived plasmids together with pHMG-PB1 (0.5 μg), pHMG-PB2 (0.5 μg), pHMG-PA (0.5 μg), and pHMG-NP (1 μg) plasmids. Cells were incubated at 37°C until harvest at 18, 24, or 48 h posttransfection. Using the CAT ELISA Kit (Roche), cell extracts were prepared in 250 μl of the lysis buffer provided in the kit and tested for CAT levels. This procedure allowed detection of 0.05 ng/ml CAT. All experiments were done in duplicate.

RNA analysis

Total RNAs were prepared from COS-1 cells at 24 h posttransfection using the Trizol reagent (Gibco-BRL) and analyzed by Northern blot. The RNA samples were run on a 1.2% agarose, MOPS 1×, and 0.6 M formaldehyde gel, blotted onto a nylon membrane (Hybond N, Amersham), and fixed by UV irradiation. Membranes were hybridized with a CAT-specific ^{32}P-labeled riboprobe, washed three times in 0.1× SSC, 0.1% SDS at 75°C for 15 min, dried, and exposed on a STORM820. Quantification was obtained using the Image Quant program (Molecular Dynamics). For detection of the viral-like mRNAs, poly(A)$^+$ RNAs were purified from total RNAs using the Gen-Elute mRNA Miniprep Kit (Sigma) and were analyzed by slot blotting as described previously (Naffakh *et al.*, 2001).

Sequencing of the 3′ end of genomic RNA segments

Virus isolates PR8 and MAL were grown in 11-day-old embryonated chicken eggs. Viral genomic RNA was extracted using the Trizol reagent (Gibco-BRL) and submitted to a polyadenylation reaction for 30 min at 30°C using the yeast poly(A) polymerase (Amersham Pharmacia Biotech) in the presence of 10 mM ATP. Complementary DNAs were prepared by reverse transcription of the polyadenylated RNAs using a $(dT)_{15}$ oligonucleotide as a primer and 20 U AMV reverse transcriptase (Promega) with incubation at 42°C for 1 h followed by incubation at 55°C for 15 min. Amplification was performed using an anchored $(dT)_{15}$ oligonucleotide (5′-AGATGAATTCGGTACC $(T)_{15}$-3′) together with a primer of negative polarity specific for the coding sequences of each of the eight segments of vRNA located about 300 bp upstream of the 3′ end. The AmpliTaq DNA Polymerase (Perkin–Elmer) was used in the presence of 10 mM $MgCl_2$. Thirty-five cycles were performed, each consisting of 10 s at 94°C, 30 s at 40°C, and 30 s at 72°C. The PCR products were purified using a PCR Purification Kit (Qiagen) and sequenced with an internal oligonucleotide, using the protocol described above. The exact sequences of the primers used for amplification and sequencing of the 3′ end of each segment can be obtained from the authors upon request.

Sequencing of the 5′ end of genomic RNA segments

Viral genomic RNA was submitted to reverse transcription as described above, using as primers oligonucleotides of positive polarity specific of the coding sequences of each of the eight segments and located around 400 bp upstream of the 5′ end. The cDNAs were purified using a PCR Purification Kit (Qiagen) and submitted to an elongation reaction for 45 min at 37°C with the *Escherichia coli* Terminal Deoxynucleotide Transferase (Amersham Pharmacia Biotech) in the presence of 5 mM dATP. A first round of amplification was performed using the anchored $(dT)_{15}$ oligonucleotide described above, together with an oligonucleotide of positive polarity specific for the coding sequence of each of the eight segments of vRNA, located about 300 bp upstream of the 5′ end. A second round of that "half-nested" reaction was performed after a 1:25 dilution step, using more proximal segment-specific primers together again with the anchored $(dT)_{15}$ primer. The conditions used for amplification were the same as described above for determination of the vRNA 3′ ends. The PCR products were purified using a PCR Purification Kit (Qiagen) and sequenced with an internal oligonucleotide, using the protocol described above. The exact sequences of the primers used for amplification and sequencing of the 5′ end of each segment can be obtained from the authors upon request.

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Efficient formation of influenza virus-like particles: dependence on the expression levels of viral proteins

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It has previously been demonstrated in this laboratory that an influenza virus-like chloramphenicol acetyltransferase (CAT) RNA could be expressed in COS-1 cells that synthesized all ten influenza A virus-encoded proteins from recombinant plasmids. It was also shown that supernatant fluids harvested from these cultures contained virus-like particles (VLPs) that could deliver an enclosed CAT RNA to MDCK cells. Here, it is shown that the levels of expression of the reporter gene in the COS-1 and/or MDCK cells can be altered drastically by modifying the concentrations of the recombinant plasmids transfected in the COS-1 cells. Thus, it was observed that overexpression of NS2 reduced CAT expression in COS-1 cells, whereas overexpression of M2 and NS1 proteins dramatically decreased transmission of the CAT RNA to the MDCK cultures. These results are discussed with reference to the roles of these proteins during virus replication. From these experiments, a ratio of transfected plasmids was found that increased the efficiency of the previously described system by 50–100-fold. Under these optimized conditions, it was demonstrated that VLPs can be formed in the absence of neuraminidase expression and that these VLPs remained aggregated to each other and to cell membranes. Moreover, it is shown that CAT RNA transmission was dependent on specific interactions of the ribonucleoprotein complex with other viral structural polypeptides. These data demonstrate the usefulness of this encapsidation–packaging system for the study of different aspects of the influenza virus life-cycle.

Introduction

Influenza A virions are pleomorphic, enveloped particles with a diameter of 80–120 nm. The viral genome, which consists of eight negative-sense, single-stranded RNAs, has a coding capacity for ten polypeptides. The virion contains three integral membrane proteins, haemagglutinin (HA), neuraminidase (NA) and the M2 ion channel protein. Six other viral proteins are found within the virion membrane. Four of them [nucleoprotein (NP), PB1, PB2 and PA] are associated with the viral genome to form ribonucleoprotein (RNP) complexes and the other two polypeptides, M1 and NS2 [also called NEP; O'Neill *et al.* (1998)], interact with each other and with the RNPs. The NS1 protein is the only non-structural component of the virus (Lamb, 1989; Lamb & Krug, 1996).

The virus is internalized by receptor-mediated endocytosis and, after fusion of the viral and endosomal membranes, the infecting RNPs are transported from the cytosol to the cell nucleus (Martin & Helenius, 1991), where replication and transcription of the viral genome takes place (Herz *et al.*, 1981). The newly synthesized RNPs are then exported from the cell nucleus to the cytoplasm (Martin & Helenius, 1991; Whittaker *et al.*, 1996; O'Neill *et al.*, 1998) and should reach the proximity of the cellular membrane, where virus budding occurs (Compans & Dimmock, 1969).

Although the interactions between the virus components that govern formation of virion particles are poorly understood, it is thought that contacts between the RNPs and other virus components are critically important. In fact, it has been shown that interactions between the RNPs and the M1 and NS2 proteins modulate the nuclear–cytoplasmic transport of RNPs (Martin & Helenius, 1991; Whittaker *et al.*, 1996;

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O'Neill *et al.*, 1998) and morphological (Murti *et al.*, 1992) and biochemical (Zvonarjev & Ghendon, 1980; Zhirnov, 1992) observations suggest that M1 protein associates with the RNPs in the virion. Despite these reports, functional evidence demonstrating the importance of the interactions between RNPs and other virus factors for the formation of infectious particles is still lacking.

Contacts between the cytoplasmic tails of the virus membrane proteins and the virion internal components are also important for formation of the budding particle. Thus, viruses lacking the cytoplasmic tail of HA or NA or both have reduced infectivity and show alterations in their morphology (Jin *et al.*, 1994, 1997; García-Sastre & Palese, 1995; Mitnaul *et al.*, 1996). The role of NA in the formation of infectious virions has been the subject of a number of studies. It has been shown that NA-deficient viruses produce particles that form large aggregates on the cell surface (Palese *et al.*, 1974; Liu *et al.*, 1995) and that these particles, when released from the cell, can complete a round of replication in animals and cell culture (Liu *et al.*, 1995). These results indicate, therefore, that NA activity is needed to prevent formation of virus aggregates and to allow the release of fully assembled virus particles from the cell surface. It should be mentioned that the NA-deficient mutants, which were selected in the presence of bacterial NA, contained a deleted NA segment that retains the capacity to code for an N-terminal NA peptide (Yang *et al.*, 1997). It is, however, not known whether expression of this N-terminal sequence, which includes the membrane-anchoring region of NA, is required for formation of virions.

Recently, we described a system in which a synthetic influenza A virus-like chloramphenicol acetyltransferase (CAT) RNA could be encapsidated, replicated and packaged into virus-like particles (VLPs) in cells expressing all virus-encoded polypeptides from plasmids (Mena *et al.*, 1996). This system is analogous to those described for the negative-sense RNA viruses vesicular stomatitis virus (Pattnaik & Wertz, 1991), rabies virus (Conzelmann & Schnell, 1994), Bunyamwera virus (Bridgen & Elliott, 1996) and human respiratory syncytial virus (Teng & Collins, 1998). The influenza virus rescue system was, however, very inefficient with respect to transmission of the CAT RNA by the VLPs. Therefore, the system was not suitable for systematic studies of the roles played by the viral proteins during virus replication.

Here, we describe the optimization of the rescue system by a factor of $\sim$ 50–100-fold and show the usefulness of this system for the study of the role of NA in formation of VLPs and to demonstrate that interactions of RNP components with other virus factors are required for formation of functional VLPs.

Methods

■ **Cell cultures and viruses.** COS-1 and MDCK cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum. Recombinant vaccinia virus (vTF7-3), which expresses T7 RNA polymerase, was kindly provided by B. Moss (National Institutes of Health, Bethesda, MA, USA) (Fuerst *et al.*, 1986). The influenza virus strains used were A/Victoria/3/75 (H3N2), A/Puerto Rico/8/34 (H1N1) and B/Panamá/45/90.

■ **Plasmids and RNAs.** Plasmids pGEM-PB1, pGEM-PB2, pGEM-PA, pGEM-NP, pGEM-HA, pGEM-NA, pGEM-M1, pGEM-M2, pGEM-NS1 and pGEM-NS2, encoding the influenza virus polypeptides PB1, PB2, PA, NP, HA, NA, M1, M2, NS1 and NS2, respectively, from the A/Victoria/3/75 strain, have been described previously (Mena *et al.*, 1994, 1996). In these plasmids, the virus genes are cloned downstream of the T7 promoter of plasmids pGEM-3 or pGEM-4 (Promega). Plasmid pGEM-M3-1, which contains a cDNA copy of $mRNA_3$ (derived from the M segment) (Lamb *et al.*, 1981) cloned under the control of the T7 promoter of plasmid pGEM-3, was derived by RT–PCR from mRNA isolated from cells infected with influenza virus A/Victoria/3/75. Plasmids pGB-PB1-89.1, pGB-PB2-2, pGB-PA-4 and pGB-NP-7, encoding the influenza virus proteins PB1, PB2, PA and NP, respectively, from the B/Panamá/45/90 strain, have been described previously (Jambrina *et al.*, 1997). Plasmids pIVACAT1/S (Piccone *et al.*, 1993) and pT7NSBCAT (Barclay & Palese, 1995) were kindly provided by P. Palese and W. S. Barclay (Mount Sinai School of Medicine, New York, USA). These plasmids were used to generate influenza A and B virus-like model RNAs after transcription with T7 RNA polymerase. These RNAs contain the CAT gene in negative polarity flanked by the 5′ and 3′ untranslated sequences of the RNA segment encoding the NS proteins of the corresponding influenza A or B virus. Plasmid concentrations were estimated spectrophotometrically by assuming that an A_{260} of 1 corresponded to 50 μg/ml DNA.

■ **Antibodies and immunoblotting.** Monoclonal antibodies (MAbs) M/58/p51/G, HA1-50 and M/234/1/F4, which recognize the A/Victoria/3/75 NP and HA proteins, and a rabbit antiserum that recognizes the C terminus of NP have been described previously (Arrese & Portela, 1996; López *et al.*, 1986; Sánchez-Fauquier *et al.*, 1987). Goat antiserum against M2 protein was a gift from Alan Hay (National Institute for Medical Research, London, UK). A polyclonal antiserum against M1 protein was obtained by immunizing rabbits with a denatured, histidine-tagged M1 protein. Western blotting analysis with antisera to M2, M1 or NP and MAb HA1-50 was carried out as described previously (Arrese & Portela, 1996).

■ **Construction of plasmids pGEM-M1Δ, pGEM-M2Δ and pGEM-NS2Δ.** Plasmid pGEM-M2 contains a cDNA copy of the M2 mRNA cloned in the polylinker of plasmid pGEM-3 (Promega). In this plasmid, the nucleotide sequence following the T7 promoter is GGGAGACCGGAATTCGAGCTCGGTACCCTCTTCAagcaaacgcag-gtagatatcgaaag*atg* ..., where the pGEM-3 vector sequence is shown in capitals and the influenza virus-derived sequence is shown in lower case. The *Eco*RI site present in the vector is underlined and the ATG initiation codon of the M2 protein is indicated in italics. A new *Eco*RI site was introduced in this plasmid by converting the sequence ta into at. This mutagenesis step was carried with the Transformer site-directed mutagenesis kit (Clontech). The mutagenized plasmid was then digested with *Eco*RI and circularized to yield plasmid pGEM-M2Δ. This plasmid contains the sequence GGGAGACCGGAATTCgaaag*atg* ... following the T7 promoter and therefore lacks most of the virus sequences present upstream of the ATG codon in plasmid pGEM-M2.

An analogous strategy was used to generate plasmids pGEM-M1Δ and pGEM-NS2Δ from plasmids pGEM-M1 and pGEM-NS2, respectively. The only difference was that an *Xba*I site, instead of the *Eco*RI site, was introduced to prepare plasmid pGEM-M1Δ, since plasmid pGEM-M1 was derived from the pGEM-4 vector. The sequences downstream of the T7 promoter in these plasmids were GGGAGACCGGAAGCTT-

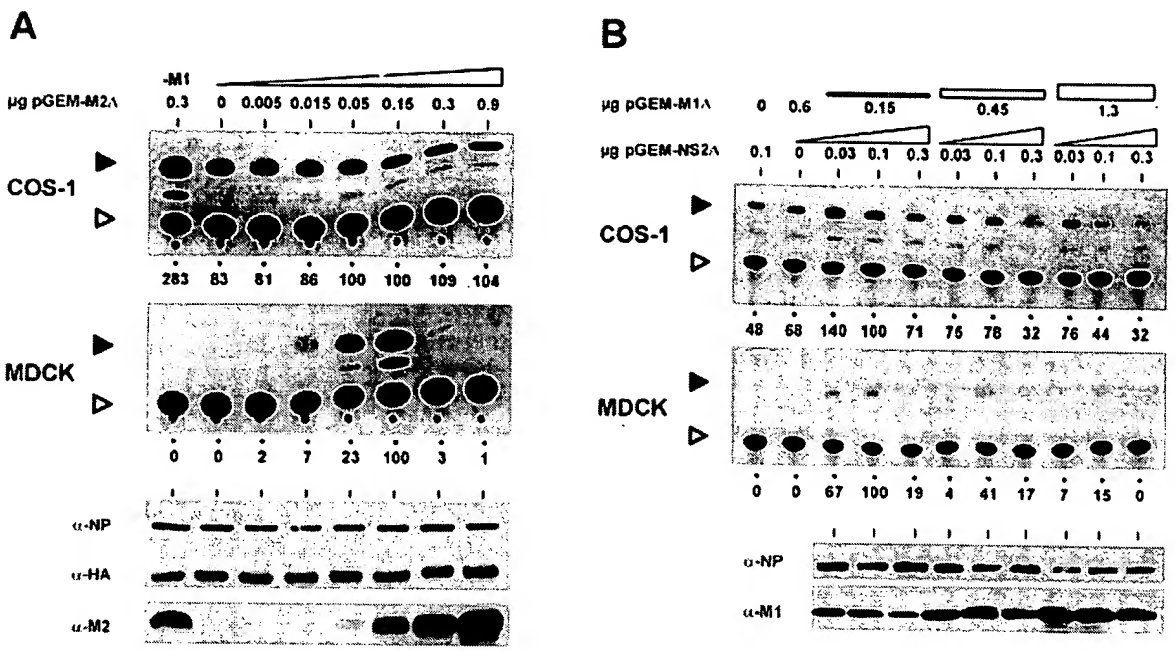

Fig. 1. Optimization of input amounts of plasmids pGEM-M2Δ, pGEM-M1Δ and pGEM-NS2Δ. (A) COS-1 cells were infected with vTF7-3 and transfected with plasmids encoding proteins PB1, PB2, PA, NP, HA, NA, M1 and NS2 (the amounts are indicated in the text) and the indicated amounts (μg) of plasmid pGEM-M2Δ. The cultures were transfected 5 h later with a synthetic CAT RNA. The supernatants from these cultures were harvested 72 h post-infection and added to MDCK cells that were superinfected with a helper influenza virus as detailed in Methods. Aliquots of the COS-1 and MDCK cell extracts were assayed for CAT activity and autoradiographs of the corresponding TLC plates are shown in the figure, with the positions of [^{14}C]chloramphenicol and 3-acetylated [^{14}C]chloramphenicol indicated by open and filled arrowheads, respectively. CAT activities in MDCK cells were expressed as a percentage of that obtained in the sample yielding the highest activity (as shown bellow each lane on the TLC). CAT activities in COS-1 cells were expressed as a percentage by considering the activity of the COS-1 cell sample corresponding to that yielding the highest CAT level in MDCK cells as 100%. The relative CAT activities are shown below each of the samples of the corresponding TLC plate. The lower panel corresponds to a Western blot of the COS-1 cell extracts probed with antibodies (α) against the proteins indicated. In sample −M1, the plasmid pGEM-M1Δ was not included in the transfection mixture. (B) The experiment was performed as described in (A) except that the amount of plasmid pGEM-M2Δ included in the transfection mixture was 150 ng and the concentrations of pGEM-M1Δ and pGEM-NS2Δ were varied as indicated.

GCATGCCTGCAGGTCGACTCTAGAaag*atg* ... (pGEM-M1Δ) and GGGAGACCGGAATTCgacata*atg* ... (pGEM-NS2Δ).

■ **Assay for detection of functional VLPs.** The standard protocol was carried out basically as described by Mena *et al.* (1996). The major modifications were (i) that plasmids pGEM-M1Δ, pGEM-M2Δ and pGEM-NS2Δ were used instead of plasmids pGEM-M1, pGEM-M2 and pGEM-NS2 and (ii) that the influenza virus strain A/Puerto Rico/8/34 substituted for A/Victoria/3/75 as helper virus, since the former grows to higher titres in cell culture. Briefly, the protocol was as follows. Cultures of COS-1 cells (10^6 cells) growing in 35 mm diameter dishes in the presence of DMEM-Ara-Ant (DMEM containing 40 μg/ml cytosine β-D-arabinofuranoside, 100 U/ml penicillin and 100 μg/ml streptomycin) were infected with vTF7-3 (m.o.i. of 5). After virus adsorption, the cultures were incubated with 1 ml DMEM-Ara-Ant that was supplemented with a 100 μl mixture that contained cationic liposomes and the plasmids indicated in each case. After the optimization experiments, the optimal amounts of the plasmids in the transfection mixture were: pGEM-PB1 (0·6 μg), pGEM-PB2 (0·6 μg), pGEM-PA (0·2 μg), pGEM-NP (2 μg), pGEM-HA (0·6 μg), pGEM-NA (0·6 μg), pGEM-M1Δ (0·15 μg), pGEM-M2Δ (0·15 μg) and pGEM-NS2Δ (0·1 μg) (see text for details). After 5 h incubation with the plasmids, the cells were transfected again with a mixture containing 0·5 μg synthetic CAT RNA. After incubation overnight, the medium was replaced with 1 ml DMEM-Ara-Ant and the cultures were incubated for an additional 48 h. Cell supernatants were then harvested and cell extracts were prepared. Aliquots of the cell extracts were used for Western blotting and for determination of CAT activity (see below).

The supernatant collected from the COS-1 cells was clarified by centrifugation for 15 min in a microcentrifuge and subjected to three cycles of freezing and thawing. To test for the presence of VLPs, an aliquot of the supernatant (typically 400 μl) was incubated with trypsin (2·5 μg/ml) for 15 min at 37 °C and added to 10^6 MDCK cells, growing in 35 mm diameter dishes in the presence of DMEM-Ara-Ant. After 1 h incubation, the cells were superinfected with influenza virus A/Puerto Rico/8/34 (or B/Panamá/45/90 when indicated) at an m.o.i. of 5 and the cultures were incubated for 17 h. Cell extracts were then prepared for determination of CAT activity. Routinely, for these assays a sample corresponding to 5000 COS-1 cells and to 50000 MDCK cells was incubated for 1 h at 37 °C with [^{14}C]chloramphenicol as described previously (Mena *et al.*, 1996). Quantification of CAT activity was performed by phosphorimaging of the acetylated spots detected on TLC plates by using a Fujix Bas 1000 phosphorimager and the software PCBAS v2.09.

■ **Electron microscopy.** Transfected cultures were pre-cooled for 15 min at 4 °C and incubated for 30 min with the anti-HA MAb M/234/1/F4 (diluted 1:5 in DMEM containing 5% sheep serum). After washing with DMEM–5% sheep serum, the samples were incubated for 30 min with a secondary antibody (10 nm gold-labelled, goat anti-mouse IgG; Auroprobe EM GAM IgG G10; Amersham) diluted 1:20 in DMEM. After washing with Tris-buffered saline (25 mM Tris–HCl, pH 7·4, 2·68 mM KCl, 137 mM NaCl), the cells were fixed with 2% glutaraldehyde in 0·1 M cacodylate buffer (pH 7·4) and post-fixed with 1% osmium tetroxide prepared in the same buffer for 1 h at 4 °C. The fixed cells were then dehydrated and embedded in epoxy resin EPON 812. Sections prepared with an LKB Ultratome IV were post-stained in 1% aqueous uranyl acetate for 15 min and then 3 min in lead citrate and were visualized with a Philips 400T electron microscope at 80 kV.

Results

Optimization of the CAT rescue system

As detailed previously (Mena *et al.*, 1996), the protocol followed to generate and detect recombinant VLPs included two steps. In the first step, COS-1 cells were infected with vTF7-3 and transfected with the pGEM-derived plasmids encoding all influenza virus polypeptides and with a synthetic, negative-sense CAT RNA. In the second step, the supernatant fluids collected from the transfected COS-1 cells were treated with trypsin and incubated with fresh MDCK cultures that were then superinfected with a wild-type influenza helper virus. Detection of CAT activity in COS-1 cells demonstrated expression of the model CAT RNA by the recombinant polymerase, whereas detection of CAT activity in the MDCK cell extracts indicated that the COS-1 cell supernatant contained functional VLPs, i.e. VLPs competent to deliver an enclosed CAT RNA into MDCK cells.

It has been shown for a number of systems in which a model RNA is replicated and/or packaged that obtaining the highest replication and/or packaging levels depends on finding the optimal ratio of transfected plasmids (Pattnaik & Wertz, 1990; Dunn *et al.*, 1995; Sanz-Ezquerro *et al.*, 1995; Teng & Collins, 1998). It was therefore decided to test the concentration-dependent effects of the transfected plasmids on the formation of influenza VLPs by following the protocol outlined above. The experiments were initiated with only the nine plasmids that encode the viral structural components, since we had demonstrated previously that formation of VLPs does not require expression of NS1 protein (Mena *et al.*, 1996). The concentrations of the plasmids encoding the virus core proteins were those found previously to be optimal for expression of a synthetic CAT RNA (Sanz-Ezquerro *et al.*, 1995) and the starting concentrations of the remaining plasmids were those used in the previous report (1 μg of each plasmid).

The first experiment was to optimize the concentration of plasmid pGEM-M2Δ (Fig. 1A), since preliminary assays indicated that the concentration of the M2 protein drastically affected the CAT activity detected in MDCK cells (not shown). It was observed that increasing the amount of transfected M2 plasmid resulted in greater accumulation of the M2 protein in COS-1 cells, as expected from the transient expression system used (Fuerst *et al.*, 1986). To detect alterations in the expression levels of the other recombinant proteins, which could arise as a result of transfecting different doses of a particular plasmid, we routinely checked for the accumulation of NP and/or HA in the different cell samples. As can be observed in Fig. 1(A), there were no significant changes in the accumulation of these two proteins in any of the samples analysed. Moreover, the reporter gene activity measured in COS-1 cells was not affected by the level of expression of M2, indicating that this protein does not modify the functionality of the core proteins to express the input CAT RNA. However, the concentration of the M2 plasmid affected formation of functional VLPs drastically. Transfection of very small amounts of the plasmid (5 ng) were sufficient to allow detection of functional VLPs and maximum detection was achieved after transfection of 150 ng plasmid. From that point on, CAT activity in MDCK cells decreased until it reached virtually background values with transfection of 0·9 μg plasmid. On the basis of these results, all subsequent transfection experiments were carried out with 150 ng pGEM-M2Δ.

It was next decided to titrate the plasmids encoding the M1 and NS2 proteins. In preliminary tests, it was observed that transfecting more than 300 ng pGEM-NS2Δ had an inhibitory effect on CAT expression in COS-1 cells (data not shown, see below), whereas transfection of small amounts of the M1 plasmid (50 ng) resulted in low levels of CAT expression in the MDCK cultures (data not shown). Since M1 and NS2 proteins appear to interact with each other (Yasuda *et al.*, 1993; O'Neill *et al.*, 1998), it was decided to carry out co-transfection experiments with the plasmids encoding these two proteins. On the basis of preliminary experiments, the doses chosen for these analyses varied from 150 to 1300 ng for pGEM-M1Δ and from 30 to 300 ng for pGEM-NS2Δ (Fig. 1B). For each of the three doses of pGEM-M1Δ tested, 100 ng of the NS2 plasmid always yielded the highest CAT activities in MDCK cells; the sample transfected with the smallest amount of M1 plasmid was the one which yielded the highest reporter gene activity in MDCK cells. It was again observed that there was a direct correlation between the amount of plasmid pGEM-M1Δ transfected and the concentration of M1 protein in the COS-1 cultures. The accumulation of NS2 protein was not tested because of the lack of an appropriate immunological reagent.

Once the optimal amounts of the M1 (150 ng) and NS2 (100 ng) plasmids were determined, the dose-dependent effects of HA and NA plasmids in the CAT rescue system were investigated. As shown in Fig. 2(A), the different amounts of plasmids tested did not have any significant effect on the CAT activity obtained in COS-1 cells and the doses of these two plasmids that yielded the highest level of CAT expression in MDCK cells were 600 ng.

It was then decided to study the effect of NP concentration on the system and to confirm the inhibitory effect (mentioned

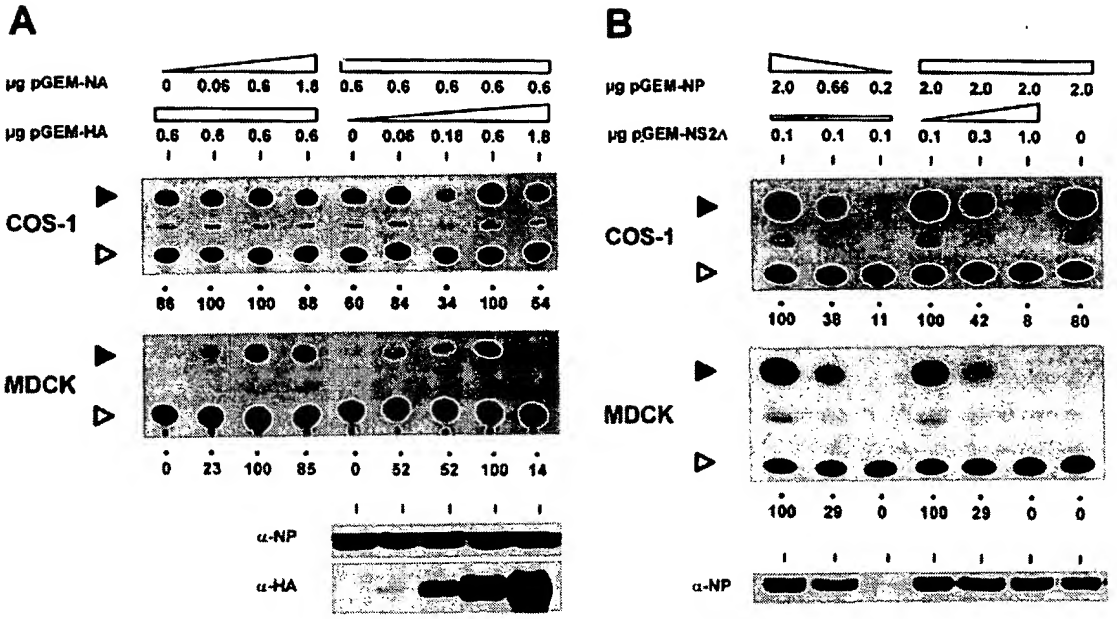

Fig. 2. Optimization of input amounts of plasmids pGEM-HA, pGEM-NA, pGEM-NP and pGEM-NS2Δ. (A) The experiment was performed as described in Fig. 1 (B) except that the amounts of plasmids pGEM-M1Δ and pGEM-NS2Δ included in the transfection mixture were 150 and 100 ng, respectively. The concentrations of pGEM-HA and pGEM-NA were varied as indicated. (B) The experiment was performed as described in (A) except that the amounts of pGEM-HA and pGEM-NA included in the transfection mixture were 600 ng each. The concentrations of plasmids pGEM-NP and pGEM-NS2Δ were varied as indicated.

above) of NS2 on expression of the CAT RNA in COS-1 cells (Fig. 2B). It was observed that reducing the amount of NP plasmid led to a reduction in the level of CAT expression in COS-1 cells. A similar effect was observed by increasing the amount of plasmid pGEM-NS2Δ in the transfection mixture. In both cases, the CAT activity detected in MDCK cells was roughly proportional to that detected in COS-1 cells. One interpretation of these results would be that either small amounts of NP or large amounts of NS2 reduced the production of viral RNPs (containing the CAT RNA) in transfected cells. Consequently, less viral RNP would be packaged into VLPs and lower CAT activity would be detected in the MDCK cell extracts. On the basis of the results obtained, the doses of plasmids encoding NP and NS2 were maintained at 2 μg and 100 ng, respectively.

Once the system had been optimized in the absence of NS1 protein, we re-examined the effect of this protein on formation of VLPs. It was confirmed that NS1 protein was not required for efficient transmission of CAT RNA to MDCK cells (Fig. 3 A) (Mena *et al.*, 1996). Strikingly, it was observed that increasing the amount of NS1 plasmid from 100 ng to 2 μg resulted in a ~ 100-fold reduction in the CAT activity detected in MDCK cells, whereas within this dose range, there was only a ~ 3-fold reduction in the CAT activity observed in the COS-1 cell extracts.

Finally, the possible effect of $mRNA_3$ on the rescue system was tested. $mRNA_3$ is a spliced product derived from RNA segment 7 and it contains an open reading frame for a 9 amino acid peptide that has never been found in infected cell cultures (Lamb *et al.*, 1981). As shown in Fig. 3(B), expression of $mRNA_3$ did not have a significant effect on the reporter gene activities reached in COS-1 and MDCK cells. It should be pointed out that the M1 mRNA (derived from plasmid pGEM-M1Δ) will not be spliced to generate $mRNA_3$, since it lacks the 5′ splice site. Moreover, it is worth mentioning that the T7-derived transcripts produced in the vaccinia T7 system are not expected to be spliced, since they are synthesized in the cytoplasm.

From the above experiments, we determined the optimal concentration of the nine plasmids encoding the virus structural polypeptides that allowed efficient formation of functional VLPs. By considering the amounts of the MDCK cell extract tested in the CAT assays (5% as compared to 20–50% in the previous report) as well as the CAT activities observed, it was calculated that the optimized rescue system was 50–100-fold more efficient than the previous one (Mena *et al.*, 1996) for CAT transmission to the MDCK cells.

CAT transmission to MDCK cells is mediated by VLPs

Using the optimized conditions, a series of experiments identical to those described in the previous report (Mena *et al.*, 1996) were carried out to characterize the particles that transmitted the CAT RNA. It was confirmed, with three different plasmid preparations, that expression of all viral

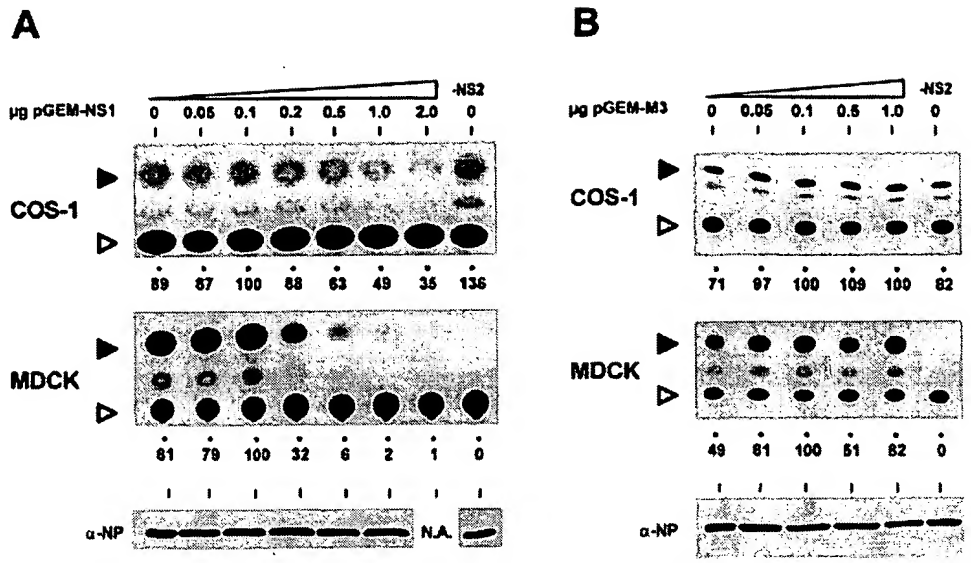

Fig. 3. Optimization of input amounts of plasmids pGEM-NS1 and pGEM-M3. The experiment was performed as described in Fig. 2 (B). The concentrations of plasmids pGEM-NS1 (A) and pGEM-M3-1 (B) were varied as indicated. N.A., Not analysed. In sample −NS2, the plasmid pGEM-NS2Δ was not included in the transfection mixture.

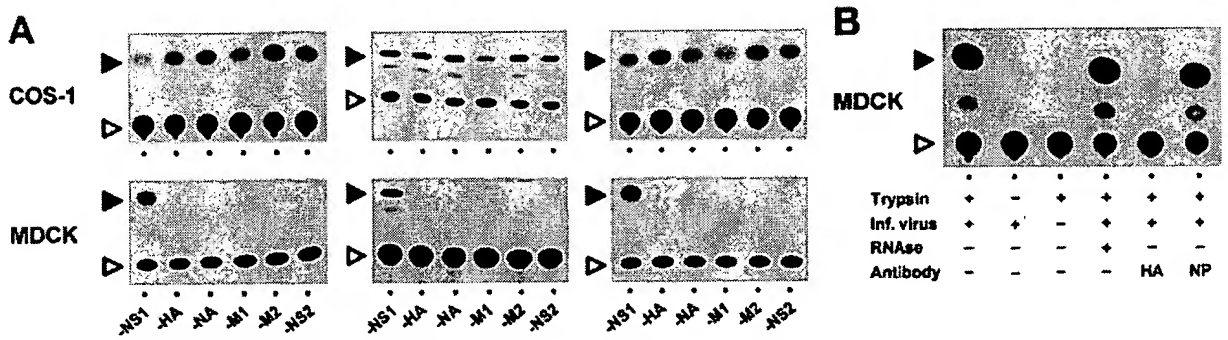

Fig. 4. Viral proteins required for transmission of CAT RNA and characterization of the VLPs that transmit the CAT RNA to MDCK cells. (A) COS-1 cells were infected with vTF7-3 and transfected with the nine plasmids encoding the structural viral polypeptides (sample −NS1) or with only eight of them (samples −HA, −NA, −M1, −M2 and −NS2, where the plasmid encoding the indicated gene product was also omitted). The cultures were then transfected with a CAT RNA and the supernatants from these cultures were tested on MDCK cells. Aliquots of the COS-1 and MDCK cell extracts were assayed for CAT activity as detailed in Methods. (B) The supernatant obtained from COS-1 cells expressing the nine structural virus polypeptides and transfected with a CAT RNA was harvested. Aliquots of this supernatant were then incubated with trypsin, RNase A or MAbs specific for influenza virus HA (MAb 234/1/F4) or NP (MAb M58/p51/G) as indicated. The treated supernatants were next added to MDCK cells, which were then mock-infected or infected with the influenza virus strain A/Puerto Rico/8/34 as indicated (Inf. virus). Cell extracts were prepared and assayed for CAT activity as described above.

structural proteins was required for detection of CAT activity in MDCK cells (Fig. 4A). Moreover, it was demonstrated that treatment of the COS-1 cell supernatant with trypsin and superinfection with an influenza helper virus were absolute requirements for detection of reporter gene activity in MDCK cells (Fig. 4B). In addition, it was shown that transmission of the CAT RNA to MDCK cells could be abolished by incubation of the COS-1 cell supernatant with a neutralizing anti-HA MAb but that it was unaffected by treatment of this supernatant with a MAb to NP, an antiserum to vaccinia virus or RNase A (Fig. 4B and data not shown). Taken together, these results allowed us to conclude that the CAT activity detected in the MDCK cell extracts was transmitted by VLPs enclosing the CAT RNA and that these VLPs should resemble authentic influenza virions, since expression of all virus structural proteins was required for CAT RNA transmission.

Electron microscopy of VLPs

In an attempt to visualize the recombinant VLPs, thin sections of COS-1 cells expressing all virus structural proteins were incubated with an anti-HA MAb and examined by

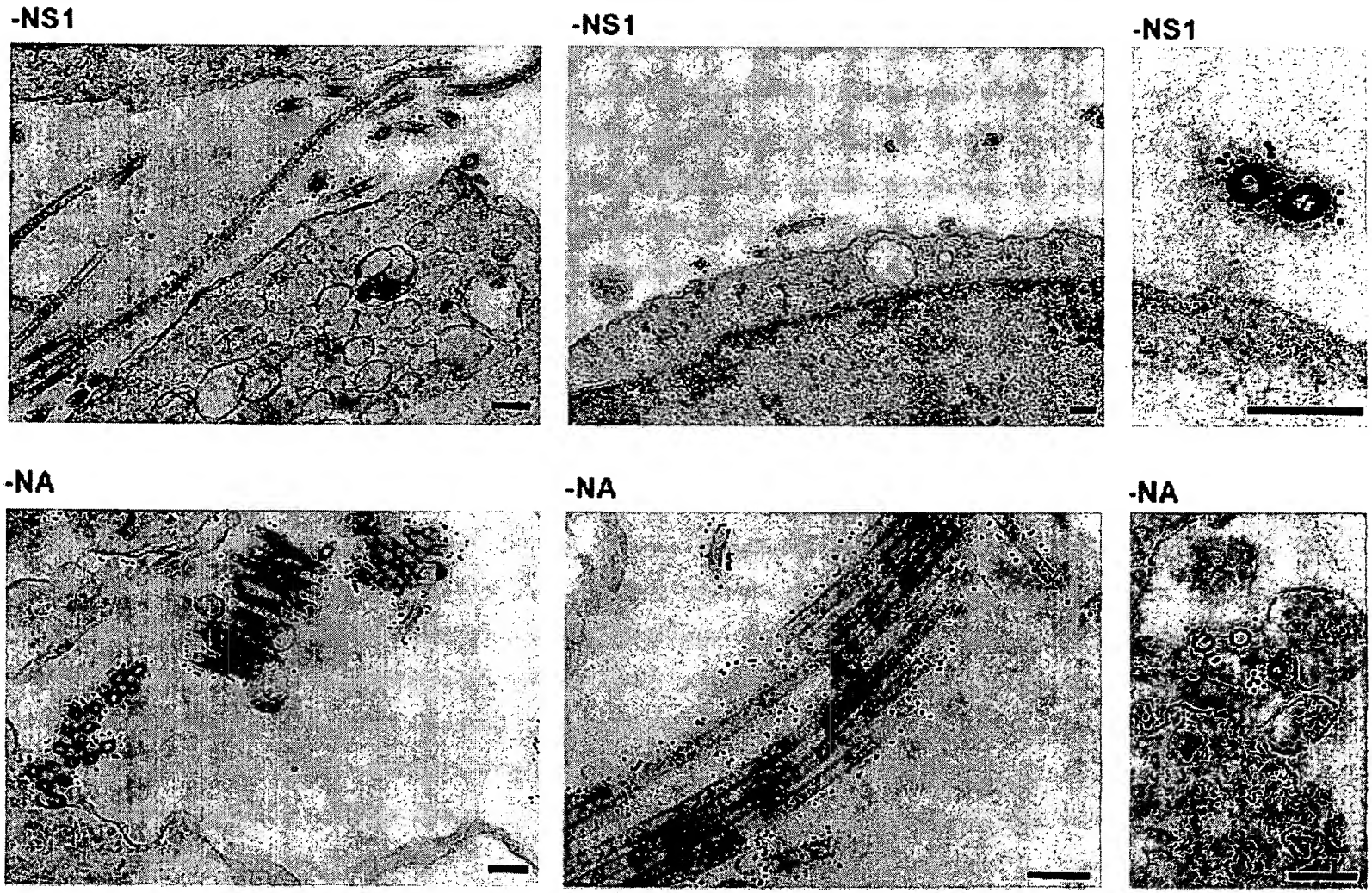

Fig. 5. Immunoelectron microscopy of transfected COS-1 cells. COS-1 cells were infected with vTF7-3 and transfected with the nine plasmids encoding all the influenza virus structural proteins (−NS1) or with a similar mixture that also lacked the NA plasmid (−NA). At 60 h post-infection, cells were incubated with an anti-HA MAb (M234/1/F4) and decorated with a 10 nm immunogold conjugate before fixation as described in Methods. Bars represent 200 nm.

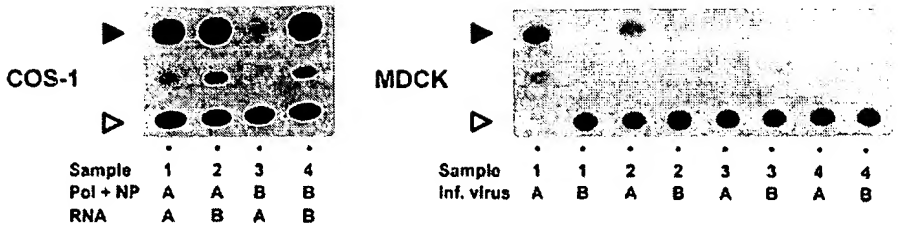

Fig. 6. Formation of heterotypic VLPs. Four COS-1 cell cultures (samples 1–4) were infected with vTF7-3 and then transfected with DNA mixtures containing the pGEM-derived plasmids corresponding to the influenza A virus proteins HA, NA, M1, M2 and NS2. The DNA mixtures contained, in addition, four plasmids encoding the three subunits of the polymerase and NP from either A/Victoria/3/75 (A) or B/Panamá/45/90 (B) influenza viruses (as indicated under Pol+NP). The amounts of type B plasmids included in the transfection mixture were: 1 μg of pGB-PB1-89.1, 0·5 μg of pGB-PB2-2, 0·5 μg of pGB-PA-4 and 3 μg of pGB-NP-7. The cells were transfected again 5 h later with an NS-CAT RNA containing the conserved ends of either type A or B virus (indicated in RNA). At 72 h post-infection, cell supernatants were harvested and the COS-1 cell extracts were assayed for CAT (left panel). Supernatants from COS-1 samples 1–4 were divided into two aliquots and added to MDCK cultures that were then infected with either influenza virus A/Puerto Rico/8/34 (A) or B/Panamá/45/90 (B) as indicated (Inf. virus). Cell extracts were prepared 17 h later and assayed for CAT expression (right panel).

electron microscopy (Fig. 5, samples −NS1). A few filamentous particles were observed in the proximity of 5–30% of the cells examined (routinely more than 100) and similar structures were not observed in cultures that did not receive plasmid pGEM-M1Δ. These particles resembled authentic influenza virions in size and morphology and could be labelled specifically with an anti-HA antibody. It was therefore concluded that the particles detected corresponded to the VLPs that transmitted the CAT RNA.

We have demonstrated that the supernatant from COS-1 cells expressing all structural proteins except NA was unable to transmit the CAT RNA to MDCK cells (Fig. 4A). One explanation of this result would be that VLPs were not formed in the absence of the NA protein. Alternatively, on the basis of the results obtained with the NA-deficient viruses (see Introduction), it is possible that budded particles were formed but that they remained aggregated and attached to the surface of the COS-1 cells. To distinguish between these two possibilities, COS-1 cultures not expressing NA were examined by electron microscopy (Fig. 5, samples −NA). Particles that had apparently completed budding were observed in 5–30% of the cells examined. Virtually all particles observed were found in aggregates or associated with the cell surface singly or in small groups. The aggregated particles could be decorated with an anti-HA MAb and structures of this kind were not observed in cultures expressing all structural proteins except NA and M1. On the basis of these findings, we concluded that the filamentous, membrane-associated particles were in fact VLPs lacking NA.

Interactions between RNPs and other viral proteins are essential for formation of functional VLPs

It was then decided to test whether the rescue system could provide functional evidence of the importance of interactions between RNPs and other viral proteins for the formation of mature virions. To this end, transfection mixtures containing plasmids encoding either the influenza A or B virus core proteins and, in addition, plasmids expressing the influenza A virus HA, NA, M1, M2 and NS2 proteins were prepared. These mixtures were transfected into COS-1 cells that were then further transfected with either an influenza A or B virus-type CAT RNA. The supernatants from these cultures were then assayed for CAT RNA transmission to MDCK cells by the standard protocol, infecting the cultures with either influenza A or B virus. The results obtained in a representative experiment are shown in Fig. 6. As we have described previously (Jambrina *et al.*, 1997), both the influenza A and B core proteins were capable of replicating/amplifying the heterotypic RNAs. However, CAT expression in MDCK cells was only observed in the samples in which all recombinant proteins and the helper virus were from the type A virus, independent of the transfected CAT RNA. These results suggest that virus type-specific contacts between the RNPs and other viral polypeptides are required for formation of functional VLPs.

Discussion

We have described here an optimized system that allows efficient rescue of synthetic CAT RNA into functional VLPs. Several interesting observations were made during the optimization experiments. Overexpression of the NS2 protein had an inhibitory effect on CAT expression in COS-1 cells, suggesting that this protein reduced the level of transcription and/or replication of the input CAT RNA. It is suggested that the observed effect may involve interactions of NS2 with virus factors other than the core proteins, since other authors did not observe such an effect in cells expressing exclusively NS2 and the four core proteins (Huang *et al.*, 1990; Enami *et al.*, 1994). It remains to be tested whether the NS2 effect may have something to do with the poorly characterized role of the protein in replication of viral RNAs (Odagiri & Tobita, 1990)

and/or with the role of NS2 in transport of RNPs from the nucleus to the cytoplasm (O'Neill *et al.*, 1998).

Overexpression of M2 completely blocked CAT RNA transmission to MDCK cultures. M2 functions as an ion channel protein (Sugrue *et al.*, 1990; Pinto *et al.*, 1992) and it has been shown that, when this protein is overexpressed, the intracellular transport of co-expressed HA is inhibited and the accumulation of HA at the plasma membrane is reduced by 75–80% (Sakaguchi *et al.*, 1996; Henkel & Weisz, 1998). It is suggested that a similar effect occurs in the rescue system, so that, by overexpressing M2, the accumulation of virus membrane proteins at the plasma membrane is reduced and hence there is a drastic reduction in the number of functional VLPs produced.

The situation with NS1 is peculiar, in that the protein was not needed for formation of functional VLPs but functional VLPs were not detected when the protein was overexpressed. The NS1 protein is an RNA-binding protein that has been found associated with RNPs (Marión *et al.*, 1997) and that inhibits nucleocytoplasmic transport of RNAs, stimulates translation of viral mRNAs and modulates splicing of influenza virus mRNAs (Nemeroff *et al.*, 1998; reviewed in Lamb & Krug, 1996; Ortín, 1998). We hypothesize that, when NS1 protein is overexpressed, the CAT RNPs are sequestered into the cell nucleus and therefore cannot be packaged into VLPs.

It should be mentioned that the three proteins (NS1, NS2 and M2) that most dramatically affected CAT expression are translated in infected cells from spliced mRNAs (M2 and NS2) or from an mRNA that is a substrate for splicing (NS1) (Lamb & Lai, 1980; Lamb *et al.*, 1981). This is consistent with previous evidence indicating that splicing of viral mRNAs is tightly regulated in infected cells (Alonso-Caplen & Krug, 1991; Valcárcel *et al.*, 1991; Shih *et al.*, 1995).

We have shown here, using three different plasmid preparations, that all virus structural proteins were required for formation of functional VLPs. It should be mentioned that in our previous report (Mena *et al.*, 1996), similar results were obtained with two plasmid preparations but in the case of a third, it was observed that expression of M2 was not required for formation of functional VLPs. We found here that transfecting very small amounts of the M2 plasmid was sufficient for a positive CAT signal to be detected in the MDCK cells. It is therefore suggested that the results obtained previously with one of the plasmid preparations were probably due to minor contamination with the M2 plasmid.

It was demonstrated by electron microscopic analysis that particles similar to wild-type virions were formed in the absence of NA expression. As indicated above, the NA-deficient mutants described previously, which were selected in the presence of bacterial NA, retained the capacity to encode an N-terminal NA peptide (Yang *et al.*, 1997). Our data indicate that expression of this NA fragment is not essential for virus particle formation, although we cannot rule out that its expression could confer a selective advantage to the NA-deficient virus. It has been described that viruses lacking the cytoplasmic tail of NA have a higher tendency to be filamentous (Mitnaul *et al.*, 1996; Jin *et al.*, 1997). This observation has been made with the WSN strain, which produces primarily spherical particles. It has not been possible to determine whether a similar effect occurs with the A/Victoria/3/75 VLPs formed in the absence of NA, since both the wild-type virions and the VLPs containing NA appear mainly as filamentous viruses.

Functional VLPs were not detected when the core proteins were derived from an influenza B virus and the rest of the structural proteins from a type A virus. This result suggests strongly that critical virus type-specific interactions between the RNP components and other viral proteins take place during virion assembly. These interactions probably involve protein–protein rather than RNA–protein contacts, since an influenza B virus RNA could be transmitted to MDCK cells by the type A proteins. In the RNP complexes, the polymerase complex holds the RNA ends together, whereas NP interacts with the rest of the RNA, exposing the RNA bases to the solvent (Klumpp *et al.*, 1997). We propose that the interactions that affect RNP packaging involve the NP polypeptide, since the polymerase subunits are minor components of the RNPs. An interesting observation made during the experiments involving heterotypic virus components was that the type A CAT RNPs were expressed in MDCK cells infected with an influenza A virus but not when the cells were infected with an influenza B virus (samples 1 and 2, Fig. 6). We had hypothesized (Mena *et al.*, 1996) that the CAT RNPs delivered into MDCK cells are capable, in the absence of helper virus infection, of primary transcription, but that detection of CAT activity depends on the replication (amplification) of the CAT RNPs, mediated by the proteins provided in *trans* by the helper virus. The lack of expression of the type A CAT RNPs in influenza B virus-infected cells indicates that the influenza B virus proteins cannot switch the type A CAT RNPs from primary transcription to replication.

In summary, we have developed an efficient encapsidation–packaging system and we have presented evidence that shows its usefulness in the characterization of the roles of viral proteins during the influenza virus life-cycle.

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Eight-plasmid system for rapid generation of influenza virus vaccines

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Abstract

The antigenic variation of influenza A virus hemagglutinin (HA) and neuraminidase (NA) glycoproteins requires frequent changes in vaccine formulation. The classical method of creating influenza virus seed strains for vaccine production is to generate 6+2 reassortants that contain six genes from a high-yield virus, such as A/PR/8/34 (H1N1) and the *HA* and *NA* genes of the circulating strains. The techniques currently used are time-consuming because of the selection process required to isolate the reassortant virus. We generated the high-yield virus A/PR/8/34 (H1N1) entirely from eight plasmids. Its growth phenotype in embryonated chicken eggs was equivalent to that of the wild-type virus. By using this DNA-based cotransfection technique, we generated 6+2 reassortants that had the antigenic determinants of the influenza virus strains A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), A/teal/HK/W312 (H6N1), and A/quail/HK/G1/97 (H9N2). Our findings demonstrate that the eight-plasmid system allows the rapid and reproducible generation of reassortant influenza A viruses for use in the manufacture of vaccines.

Author Keywords: Influenza; Vaccines; Reverse genetics

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